University of Dundee

MASTER OF SCIENCE

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Award date:
2014

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NOVEL METHOD OF SELECTING HUMAN SPERMATOZOA FOR *IN VITRO* FERTILIZATION

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MSc by Research

University of Dundee

December 2013

Contents

Acknowledgements

I would like to thank Dr Sarah Martins da Silva and Professor Christopher Barratt for the great opportunity to carry out my research at the University of Dundee and Ninewells hospital. Your diligence and self-devotion set the example for me. Many thanks to my friends in the lab, your help and our discussions were very important for me.

Special thanks to my beloved wife Marina and her parents for their wisdom and patience.

I am very grateful to my parents, Dr Irina V. Zorina and Dr Alexey Y. Biryukov, for their emotional and financial support, and discussions about all things. Without your help nothing would be possible.

Particular thanks to all my colleagues in Altravita IVF clinic who have taught me about IVF.

Declaration

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The text is written in my own words and I have consulted all of the references cited in this report.

Alexey A. Biryukov

Supervisors Statement

I certify that Alexey Biryukov has fulfilled the conditions of ordinance 39 and of the relevant regulations, such that he is qualified to submit this thesis in application for the higher degree of Master of Science.

Signatures of Supervisors

Dr Sarah Martins da Silva

Professor Christopher Barratt

List of Abbreviations

ALH: Amplitude of lateral head displacement

ART: Assisted reproductive technology

BSA: Bovine serum albumin

CASA: Computer-assisted sperm analyzer

CK: Creatine kinase

FISH: Fluorescence in situ hybridization

HBA: Hyaluronan binding assay

HA: Hyaluronic acid

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HFEA: Human Fertilization and Embryology Authority

ICSI: Intracytoplasmic sperm injection

IMSI: Intracytoplasmic morphologically selected sperm injection

IVF: In vitro fertilization

IUI: Intrauterine insemination

IS: Irvine Scientific

LIN: Linearity

MSOME: Motile sperm organelle morphology examination

NCB: Non-capacitating buffer

NHS: National Health Service

NICE: National Institute for Health and Clinical Excellence

PICSI: Physiological intracytoplasmic sperm injection

ROS: Reactive oxygen species

STF: Synthetic tubal fluid

STR: Straightness

TZI: Teratozoospermia index

TUNEL: Terminal deoxynucleotidyl transferase

VAP: Average path velocity

VCL: Curvilinear path velocity

VMP: Viscous medium penetration

VSL: Straight line velocity

WHO: World Health Organization

Project Summary

Traditionally, the diagnosis of male infertility is based on semen analysis when semen volume, sperm concentration, motility and morphology are evaluated. These characteristics help to assess how many spermatozoa are capable to get through the natural barriers of the female reproductive tract such as viscous cervical mucus, the uterus and into the fallopian tubes. The more spermatozoa are released into the vagina, and the more motile they are, the more spermatozoa will be able to penetrate through the cervical mucus and potentially find the oocyte. Even the morphology of spermatozoa provides information regarding the penetration ability and fertilization potential of sperm but cannot be an absolute indicator of fertility. Thus, these characteristics can help us to predict the chances of pregnancy *in vivo* and intrauterine insemination only, but have very limited clinical value in IVF and especially in ICSI when all natural barriers for sperm selection are eliminated. From a practical point of view sperm selection for IVF is one of the most important challenges, especially in ICSI, when the embryologist must manually and quickly choose a single spermatozoon and inject it into an oocyte. Therefore, an efficient technique for sperm selection can potentially improve fertilization and pregnancy rates in IVF.

In this thesis I developed a novel physiological method of preparing and selecting spermatozoa for IVF/ICSI which is based on sperm penetration through viscous medium *in vitro* as a model of *in vivo*. The main hypothesis is that spermatozoa that are able to enter and progress through viscous medium *in vitro* have the best fertilization potential by analogy of spermatozoa that are able to penetrate through cervical mucus in female reproductive tract.

Chapter 1 Introduction

1.1 The role of the spermatozoa in fertility

Since the first successful application of *in vitro* fertilization (IVF) was achieved in 1978 (Edwards *et al*., 1980), IVF has became an effective method of infertility treatment. The World Health Organization (WHO, 2013) defines infertility as the inability to conceive a child, after two years of unprotected regular sexual intercourse. Currently, male factor is one of the main causes of infertility. According to the National Institute for Health and Clinical Excellence (NICE), in 40% of all cases, male infertility is the main reason why the couple cannot conceive a child and IVF is the only option for the couple to get pregnant (NICE, 2013). The major causes of male infertility include varicocele (25%), genital tract obstruction (15%), testicular failure (15%), cryptorchidism (14%), idiopathic (12%), genetic conditions (8%), infections (3%), ejaculatory dysfunction (3%), hormonal dysfunction (2%), immunological conditions (2%), cancer (0.5%) and systemic diseases (0.5%) (Esteves *et al*., 2011). There are several but limited methods which can help to diagnose the fertilizing potential of a male. The most common is the conventional semen analysis with the reference values provided by WHO (Table 1.1). Semen analysis is the cornerstone of diagnosis, first and an important test when sperm concentration and motility are assessed. This information helps the doctor to understand the chances of the male partner to become a father *in vivo*: the more spermatozoa are released into vagina, and the more motile they are, the more spermatozoa will be able to penetrate cervical mucus and find the oocyte (Silber *et al.*, 1989). Other semen characteristics, for example, sperm morphology provides information about penetration ability and

embryo development but cannot be an absolute indicator of fertility (Barratt *et al*., 1995; Barros *et al.*, 1983; Maettner *et al*., 2013).

Table 1.1 Semen parameters for normozoospermia changes by WHO from 1980 till 2010

Semen parameters	WHO 1980	WHO 1987	WHO 1992	WHO 1999	WHO 2010
Volume, ml	-	≥ 2	≥ 2	≥ 2	≥ 1.5
Sperm concentration, M/ml	20-200	≥ 20	≥ 20	≥ 20	≥ 15
Total sperm count, M	-	≥ 40	≥ 40	≥ 40	≥ 39
Total motility, % motile	≥ 60	≥ 50	≥ 50	≥ 50	≥ 40
Progressive motility > 25 μm/s	≥ 2 (scale 0-3)	≥ 25%	≥ 25% (grade a)	≥ 25% (grade a)	≥32% (a + b)
Vitality, % alive	-	≥ 50	≥ 75	≥ 75	≥58
Morphology, % normal	≥ 80.5	≥ 50	≥ 30	≥ 14, strict criteria	≥ 4, strict criteria
Leukocyte concentration, M/ml	< 4.7	< 1.0	< 1.0	< 1.0	< 1.0

The main option for treating male subfertility or sperm dysfunction in general is assisted reproductive technology (ART) which comprises intrauterine insemination (IUI), IVF, and intracytoplasmic sperm injection (ICSI). Patients are allocated to IUI, IVF or ICSI based on clinical indications and semen quality, but, currently, there are no strict guidelines for semen quality to choose between the methods of treatment, and usually each clinic has its own criteria for semen characteristics (Jones *et al*., 2012). The quality of spermatozoa is a significant factor that determines the success of IVF (Aitken *et al*., 1984; Donnelly *et al*.,

1998; Evenson *et al*., 2007; Lewis *et al*., 2005). However, simple semen analysis does not always provide information about paternal factor that can affect embryo development (Tesarik *et al*., 2002). Conventional semen parameters (volume, sperm concentration, motility or morphology) do not predict fertilization or blastocyst rate *in vitro* (Benchaib *et al*., 2003; Seli & Sakkas, 2005). At the same time, sperm DNA integrity or chromosomal abnormalities are not studied in semen analysis, but are very important for the diagnosis and treatment of male infertility. Paternal effect can be associated with such genetic factors as mutations, microdeletions, aneuploidy, DNA damage and chromatin fragmentation (Seli & Sakkas, 2005). Although, spermatozoa with high level of DNA damage are able to fertilize the oocytes, embryo development can be affected (Avendaño *et al.*, 2010; Seli *et al.*, 2004). Virro *et al*. (2004) reported that in couples where males had a high level of DNA fragmentation (DFI > 30%) a low blastocyst (< 30%) and pregnancy rates and high frequency of abortion were observed. The absence of correlation between DNA fragmentation and fertilization rate corresponds with the concept that paternal DNA damage is not evident before the embryo genome is activated at the third day of development (Larson-Cook *et al.*, 2003). The high level of DNA fragmentation in sperm can result in a significant decrease in blastocyst and pregnancy rates (Muriel *et al.*, 2006; Virro *et al*., 2004). Thus, simple semen analysis can help to predict the chances of pregnancy *in vivo* and IUI only, but, is not predictive in IVF or ICSI when most of the natural barriers for sperm selection are eliminated (Brasch *et al.*, 1994; Van Voorhis *et al*., 2001).

1.2 Sperm selection *in vivo*

In vivo, the success of fertilization is reliant upon successful migration of spermatozoa through the female reproductive tract. Human spermatozoa are unable to fertilize an oocyte immediately after ejaculation. Semen is typically a coagulated mass which is liquefies within 15-20 minutes of ejaculation (WHO, 2010). The liquefaction is a two-step process caused by proteolytic degradation of the proteins. The first step termed "fibrinolysis" is accompanied by splitting of the fibrin-like protein and the second step is proteolysis of the lysed fibrin-like protein, leading to the release of free amino acids and ammonia (Lundquist *et al.*, 1955).

A period of time termed capacitation is required, when sperm are undergo a complex biochemical process (Austin, 1950; Yanagimachi *et al.*, 1976). The site of female reproductive tract where capacitation takes place, has not been identified exactly, but it initiated in the cervix (Insler *et al.*, 1980; Lambert *et al.*, 1985). After ejaculation spermatozoa migrate from the vagina to the cervix, then further to the place of fertilization – ampulla of fallopian tube and only motile, morphologically normal sperm which are able to fertilize an oocyte can pass through the viscous cervical mucus (Barratt *et al.*, 1991).

Cervical mucus appears to contain a network of macromolecules with interstices that contain low-viscosity fluid – the mucus plasma. These interstices are small compared to the size of the spermatozoa (Katz & Berger, 1980; Yudin *et al.*, 1989). Therefore, sperm pass through the mucus by distorting or rupturing its macromolecular network (Katz *et al.*, 1989). As sperm contact the mucus macromolecules, the sperm surface is subjected to high resistance that can remove

decapacitation factors attached to the plasma membrane. This resistance depends on the structural characteristics of the microstructure, the viscosity of the mucus, and the size and shape of the sperm. The viscosity of cervical mucus rapidly changes during the menstrual cycle (Figure 1.1, Clift & Hart, 1953). *In vivo* the probability of conception is high if intercourse occurs from the 10th to the 16th day of the menstrual cycle (Dunson *et al*., 1999). The fertile interval corresponds to a significant rise in estrogen, which results in a decrease of cervical mucus viscosity (Billings *et al*., 1972; Katz *et al*., 1997). During the fertile interval, mucus is accessible for motile spermatozoa. At other times of the menstrual cycle the concentration of estrogen is low and viscosity of cervical mucus is too high for sperm penetration (Kesseru-Koos, 1971).

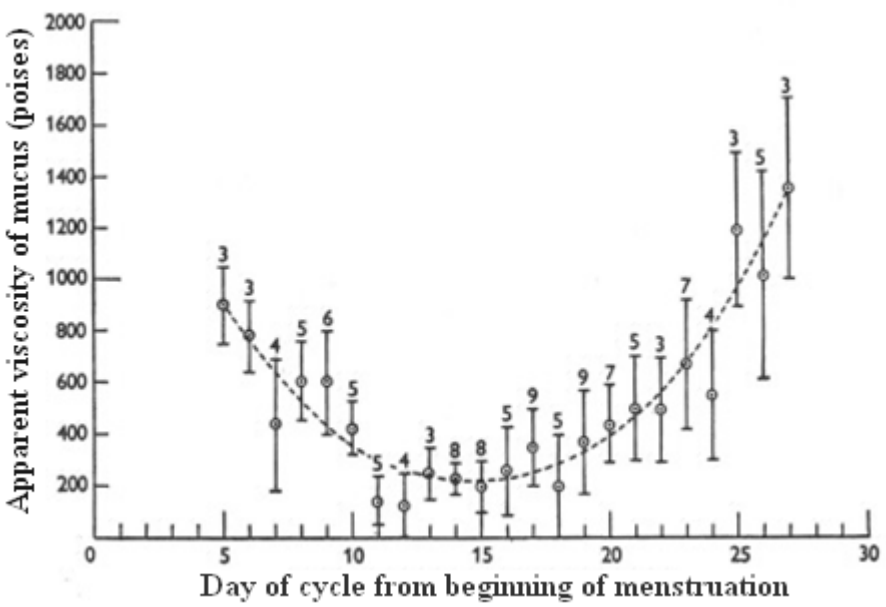


Figure 1.1 Variations in viscosity of cervical mucus in a healthy non-pregnant female. The dotted line is drawn to emphasize the main feature of the graph. The number above each point indicates the number of samples averaged. Each vertical line indicates ± standard deviation of individual readings about the mean (from Clift & Hart, 1953).

The mechanism of sperm selection in the cervical mucus is suggested to be due to sperm motility, morphology and, possibly, functionally abnormality (Pretorius *et al*., 1984). However, other factors, including mixing of the ejaculate with cervical mucus, vaginal and uterine contractions, should be considered (Fox *et al*., 1970). It is remarkable, that the components of seminal plasma may assist sperm in penetrating the vagina-cervical border. Significantly more spermatozoa were able to enter cervical mucus when a fraction of sperm was diluted 1:1 with seminal plasma than when it was diluted with Tyrode's medium, even though the sperm swam faster in the medium (Overstreet *et al*., 1980).

A few thousand spermatozoa swim into the fallopian tubes, several hundred of them reach the ampulla of fallopian tube and only one spermatozoon fuses with the oocyte (Williams *et al*., 1993). Compared to the many millions of sperm released into vagina, it is clear that selection of spermatozoa in the female reproductive tract is very stringent.

1.3 Sperm selection *in vitro*

The ejaculate consists of seminal plasma, cell debris, epithelium, and leukocytes. Spermatozoa occupy approximately 5% of the total volume of semen, and other components of semen such as seminal plasma, cell debris and leukocytes not only provide energy and protect spermatozoa from the acidic environment of the vagina, but also negatively affect the fertilization ability of sperm if exposed to semen for too long time (Aitken *et al*., 1989; Kanwar *et al*., 1979). Also, raw semen is known to be the major source of contamination in the culture system that can jeopardize the whole IVF program (Zarutskie *et al*., 1992). Therefore,

processing semen is an essential step in ART. This procedure not only isolates the fraction of the most motile spermatozoa from seminal plasma which is believed to contain decapacitation factors, but also eliminates leukocytes, cell debris and bacteria that negatively affect fertilization ability of sperm and are a source of contamination (Chang, 1957; Cottell *et al.,* 2000; Cross, 1993).

1.3.1 Swim up

The method of swim-up is based on the active movement of sperm from the liquefied semen or pre-washed cell pellet into an overlaying medium. Although this technique is distinguished by recovery of a high percentage (~90%) of motile sperm, it does not include any element of sperm selection (Aitken & Clarkson, 1988). Another major disadvantage of swim up is the fact that spermatozoa are in close contact with each other, cell debris and leukocytes, leading to production of high levels of reactive oxygen species (ROS). Due to the high concentrations of poly-unsaturated fatty acids in the sperm plasma membrane, these ROS cause lipid peroxidation and therefore potentially decrease sperm function, including motility (Aitken *et al*., 1998; Mortimer, 2000). Lastly, in swim up, ROS may lead to decrease in the percentage of normal chromatin condensed spermatozoa (Kessopoulou *et al*., 1992; Muratori *et al*., 2003) and therefore reduce the outcome of IVF treatment (Tavalaee *et al.,* 2009; Ward, 2010).

1.3.2 Density gradient centrifugation

Density gradient centrifugation (DGC) through polyvinyl pyrrolidone- or silane-coated colloidal silica particles (ø15-30 nm) in suspension is one of the most

popular methods of preparing spermatozoa for ART. Technically, this method is more complicated than swim up, because it requires different media and precise control for the time and speed of centrifugation. This method was developed in 1951 (Brakke, 1951) and later was applied to human semen (Lessley & Garner, 1983). Separation by DGC relies on differences in sperm density. Spermatozoa with morphologically normal heads have highly compacted chromatin and migrate to the high-density region of the gradient (Moustafa *et al*., 2004). The method consists of two steps: isolating sperm by density gradient medium and washing the recovered sperm. Semen is placed on the top of the density medium and then is centrifuged for 20 minutes at 300 g. After centrifugation, the supernatant is removed and the pellet is resuspended in fresh medium. It is necessary to centrifuge this sample once again to wash spermatozoa from density gradient medium. Using this technique it is possible to recover a clean fraction of highly motile spermatozoa. However, DGC also has disadvantages. Apart from the expensive media and equipment, centrifugation of sperm may be associated with membrane stress and cell damage (Makler & Jakobi, 1981; Sills *et al*., 2002). Although, the possibility of inducing damaged DNA within the embryo through the use of defective spermatozoa in IVF is still debated, the process of centrifugation may affect sperm DNA (Bungum *et al*., 2008; Sauer *et al*., 2012; Stevanato *et al*., 2008). Mahfouz *et al.* (2010) studied sperm intracellular levels of H_2O_2 and O_2 and reported that centrifugation may increase the activity of superoxide dismutase which converts the generated superoxide ion into hydrogen peroxide and potentially adversely affecting sperm quality (Burnaugh *et al.*, 2007). Lastly, this method is not very efficient for semen samples where 100% of

sperm have pin heads. The structure of these cells does not allow them to pass through the density media, although they can be very motile.

1.3.3 Migration-sedimentation

The migration-sedimentation technique was developed by Tea *et al*. (1984). This method is a combination of swim-up and a sedimentation step. A special glass or plastic tube with an inner cone is necessary to perform this method. Spermatozoa swim up from the liquefied semen into the supernatant medium and subsequently sediment in the inner cone. This method is very gentle, especially if compared with methods that require a centrifugation step, like the DGC. It is possible to obtain a fraction of highly motile and functionally competent spermatozoa. Sanchez *et al*. (1996a) demonstrated that even in cases with severe oligo- and/or asthenozoospermia, a sufficient number of motile spermatozoa can be isolated for ICSI after 2–3 hours of incubation. These authors also showed significantly higher progressive motility, normal sperm morphology, chromatin condensation and reduction of the percentage of dead spermatozoa compared with the DGC. However, the yield is very low compared with swim up and DGC. Therefore, currently this method is not widely used in everyday practice. Zavos *et al*. (2000) proposed the next generation of this method – a multi-chamber tube to retrieve functional spermatozoa for ART by means of a swim-up and sedimentation. During one hour of incubation, the sperm migrate into the overlying media in each compartment and then sediment into the peripherally situated media in each compartment. This modified method may increase the number of recovered

spermatozoa, but the assessment of its usefulness for IVF/ICSI is still to be determined.

1.3.4 Glass wool filtration

During glass wool filtration, which was described by Paulson and Polakoski in 1977, motile sperm are isolated from semen by densely packed glass wool fibers (Paulson & Polakoski, 1977). The principle of this technique lies in both the self-propelled movement of the spermatozoa and the filtration effect of the glass wool. The ejaculate is incubated in the chamber on the top of a glass wool column. Semen that has passed through the glass wool column is diluted with medium and centrifuged for 10 min at 300 g. After this centrifugation step, the pellet can be used in ART.

The efficiency of this method depends on the properties of the glass fibers. Factors such as the chemical nature of the glass (i.e. borate glass, silicate glass or quartz glass), the surface structure of the glass wool, thickness of the glass wool fibers or the pore size of the filter have to be taken into account (Ford *et al.*, 1992). The disadvantage of this method is that there is a potential risk of sperm damage because of glass wool fragments in the filtrate. Compared with the methods described above, glass wool filtration is a technique that uses the whole volume of the ejaculate and therefore yields a significantly higher total number of motile spermatozoa. It can also be used for patients with oligo- and/or asthenozoospermia (Berger *et al.*, 1985). However, after the separation of the functional spermatozoa from the immotile ones, leukocytes and debris, a centrifugation step is necessary to remove the seminal plasma. In addition to the

separation of spermatozoa, glass wool filtration has been shown to effectively eliminate leukocytes (Henkel *et al*., 1997; Sanchez *et al*., 1996b). Although it is possible to recover a clean fraction of motile sperm, this technique is not widely used because this method is not finally standardized and the commercial kit is not available.

1.3.5 Sephadex columns

In the early nineties sperm filtration by Sephadex beads was suggested (Drobnis *et al*., 1991) and a commercial sperm separation kit based on this principle (SpermPrep) became available. Compared to migration-sedimentation and swim-up, it produces significantly higher yields (Gabriel *et al*., 1993). Moreover, morphologically normal sperm cells could be enriched in the filtrate after SpermPrep separation and significantly higher pregnancy rates for intrauterine insemination as compared with the conventional swim-up method were reported (Zavos *et al*., 1992). In a comparison with DGC, SpermPrep separated sperm showed a significantly lower percentage of normally chromatin-condensed and morphologically normal spermatozoa (Hammadeh *et al*., 2001). However, the fertilization rates reported by these authors were similar. Lopez *et al*. (1993) used a prepacked PD-10 column containing Sephadex G-25 particles (Pharmacia Biotechnology, Uppsala, Sweden), which is normally used to desalt proteins in solutions, to separate human spermatozoa and compared the results with the SpermPrep method and DGC. The PD-10 column and DGC in Percoll showed similar percentages of morphologically normal sperm after preparation. However,

the SpermPrep method resulted in significantly lower values of sperm count and morphology. Thus, this method is not widely used in IVF.

1.3.6 Electrophoretic filtration system

An electrophoretic filtration method has been developed to separate spermatozoa based on electronegative charge. High quality sperm carry the greatest negative charge (Giuliani *et al.*, 2004) and can be isolated from other cells, such as leukocytes and cell debris, due to their smaller cross-sectional size (Ainsworth *et al*., 2005). Ainsworth *et al.* (2007) developed a system called Microflow CS-10 to sort sperm depending on their charge. Semen is loaded into the reservoir and after equilibration with special buffer for 5 min an electric field in the form of a constant applied current of 75 mA and a variable voltage of 18–21 V. This method of preparation is very fast as it requires only 5 min of processing, thus it could be valuable in minimizing oxidative stress to spermatozoa. A normal birth has been achieved with the sperm sorted by Microflow CS-10 (Ainsworth *et al*., 2007). However, there was no statistically significant difference in fertilization or pregnancy rates between spermatozoa isolated by Microflow CS-10 or DGC. The only advantage noted by the authors was that CS-10 was less time-consuming than DGC (Fleming *et al*., 2008). Also, it has not been demonstrated whether spermatozoa prepared by the electrophoretic filtration system are capable to fertilize the oocytes without the assistance of ICSI. Although this method is very promising, it is not offered on the market yet.

1.3.7 Microfluids

Microfluidic technology is a new, but powerful instrument in biological research (Wang *et al*., 2009). Schuster *et al*. (2003) developed a device for sperm preparation where a microfluidic technology was applied. The system consists of two parallel streams: a stream of semen and a stream of medium. Nonmotile sperm flow along their initial streamlines and exit from one outlet and motile sperm can deviate from their initial streamlines and exit through a different outlet. There is no mixing at the interface between the streams in small channels because multiple laminar streams can flow parallel to each other. It is possible to recover a fraction of highly motile (~ 98%) and morphologically normal sperm (Schuster *et al*., 2003).

Besides the special equipment is necessary to perform this method, the current device can sort only 20–40 µl of semen in one hour and it is not capable of processing an entire semen specimen. Therefore, the utility of this method is very limited.

1.4 The problem of sperm selection

Although there are several different sperm preparation methods available (Table 1.2), our knowledge about the physiological sperm selection processes in the female reproductive tract is still limited. Whether this selection is based on the special sperm characteristics or random is still controversial. Cohen and McNaughton (1974) investigated a possible selection of spermatozoa for fertilization by the female genital tract in rabbits. In that experiment, sperm recovered from rabbit uterus and washed sperm were mixed with freshly

ejaculated spermatozoa of different genotype and inseminated into the uterus of a second doe. They discovered that sperm recovered from the upper parts of the female tract were more capable to fertilize an oocyte than ejaculated or washed spermatozoa. The same effect was observed later by Fischer and Adams (1981). It was therefore concluded that part of the function of the female genital tract is the selection of sperm for fertilization. However, despite these promising results in animals, no similar experiments were carried out with humans due to ethical and technical limitations.

In most of the sperm isolating techniques the main requirement for the cell is to be motile. Information about sperm motility is important, but not enough to predict fertilization and pregnancy (Barratt *et al*., 2010; Donnelly *et al*., 1998; Takeda *et al*., 2012). Other sperm isolation methods, such as electrophoretic filtration system and DGC, which are based on sperm charge or density, are not physiological. Although pregnancies with spermatozoa prepared with these two methods were achieved, the relationship between the sperm charge or density and their fertilization capacity is not clear.

Further extensive research has evaluated other sperm parameters which are significant for fertilization and pregnancy rates. Significant correlations have been reported between the curvilinear, straight line velocities, amplitude of lateral head displacement and IVF rates (Donnelly *et al*., 1998; Hirano *et al*., 2001; Shibahara *et al*., 2004). Also, other important sperm characteristics such as apoptosis and apoptosis-like manifestations, DNA integrity, membrane maturation and ultrastructure are not directly involved in the routine sperm preparation techniques

such as swim up or DGC. Moreover, these characteristics could be affected by sperm preparation and therefore the efficiency of IVF can be reduced.

If the physiological processes of sperm selection by female reproductive tract could be replicated *in vitro*, the quality of spermatozoa used for IVF can be potentially increased.

Table 1.2 Advantages and disadvantages of different sperm preparation techniques

Method of preparing sperm for IVF	Advantages	Disadvantages
Swim up	• Clean fraction of motile sperm • Pregnancies have been achieved	• Potential risk of sperm DNA damage because of reactive oxygen species • High sperm concentration and motility are necessary
Density gradient centrifugation	• Clean fraction of motile sperm recovery • Effective for subfertile patients • Pregnancies have been achieved	• Potential risk of membrane stress and cell damage by centrifugation • Special, expensive media and equipment are required
Migration-sedimentation	• Clean fraction of motile sperm recovery • Gentle separation method	• High sperm concentration and motility are necessary • Special tubes are required • The yield is low
Glass wool filtration	• Allow processing the whole ejaculate	• Debris is present in the prepared fraction • Special tubes are required
Sephadex columns	• Clean fraction of motile sperm	• The yield is low • Special equipment is required
Electrophoretic filtration system	• Rapid method • Pregnancies have been achieved	• Equipment is not commercially available
Microfluidic technology	• Clean fraction of motile sperm	• Special chambers are required • The yield is low • Low productivity

1.5 Thesis hypothesis

Previously, in animal models, it was shown that spermatozoa recovered from the uterus and oviducts have higher fertilization rates, even when used in low concentrations, in comparison with spermatozoa recovered from the ejaculate (Cohen & McNaughton, 1974; Fischer & Adams, 1981; Siddiquey & Cohen, 1982). The new method for semen preparation and sperm selection for IVF/ICSI presented in this thesis is based on the hypothesis that spermatozoa which are able to penetrate a viscous barrier have better characteristics and therefore greater capacity to fertilize an oocyte than spermatozoa prepared by conventional methods of processing semen. This physiological technique, which is termed the viscous medium penetration (VMP) method, replicates the *in vivo* process. The spermatozoa recovered by this method can be used in IVF/ICSI without any further preparation.

1.6 Thesis aims

A series of experiments were carried out to develop and subsequently test the VMP method of processing semen and sperm selection. The experiments specifically address:

- The development of a novel physiological method for sperm selection that will allow for the recovery of sufficient number of spermatozoa for IVF.
- Evaluation of the characteristics of spermatozoa recovered by the VMP method (concentration, motility, and morphology).

- Evaluation of the sperm characteristics that influence the penetration and recovery of sperm in the VMP method.
- Comparison of the characteristics of spermatozoa recovered by VMP and swim up.
- Assessing the functional characteristics of spermatozoa recovered by the VMP method.
- Examining if the VMP method can be applied to patients with severe semen abnormalities.

Chapter 2 Materials and Methods

2.1 Semen samples

The semen samples were obtained from healthy normozoospermic donors. Sperm donors were recruited in accordance with the Human Fertilization and Embryology Authority (HFEA) Code of Practice (version 8) under the ethical approval 08/S1402/6. All donors were asked to fill out a questionnaire regarding their name, time of production, date of last ejaculation and any loss of semen (Appendix 8.1). Samples were collected by masturbation after 2 – 7 days of sexual abstinence into a clean wide mouthed plastic sterile container (Sterilin, UK). Collection pots were labeled with the donors ID number, date, and time of production. Normozoospermic donors were identified by an initial semen analysis taking into account the WHO 2010 criteria for sperm concentration (M/ml), motility (%) and morphology (%) (Table 1.1).

The semen samples from the patients were obtained from the Assisted Conception Unit, Ninewells hospital. All patients signed an informed consent prior to entry the study (Appendix 8.2). The tubes with semen were labeled with the patient ID number, date, and time of production. These tubes were delivered in the research laboratory within 10-15 minutes after the sample was examined by clinical embryologist in the Assisted Conception Unit.

2.2 Evaluating spermatozoa characteristics

2.2.1 Concentration

After liquefaction of the semen at 37° C, concentration and motility parameters of human spermatozoa were evaluated using Hamilton Thorne Computer Assisted Sperm Analysis (CASA) supplied with Olympus negative phase contrast

trinocular microscope. The cells were lightly mixed by pipetting up and down, before placing 4 µl of sperm onto a 37° C MicroCell counting slides, 20 µm depth. Slides were placed on a heated stage and left for approximately 3 minutes to obtain stable condition of a sample.

Approximately 1000 cells were counted at different fields of view from each sample population. The CASA settings were: 60 frames/second; low and high size gates: 0.35 and 2.80 respectively, low and high intensity gates: 0.5 and 2 respectively. Gates and intensity are provided to define the values within which the object can be determined as a spermatozoon. Non motile head size: 6 pixels; minimum number of data points: 13; non motile head intensity: 160.

Accuracy of evaluating sperm concentration depends on the CASA sperm detection system and precise calculation of semen volume in the field of view. The software analyzes each frame and is able to detect and exclude the objects which are larger or smaller than a sperm head. To ensure that spermatozoa were identified correctly, the CASA play back system was used. Throughout the data collection at least 95% of spermatozoa were identified per field of view. With a ×20 objective and a ×10 ocular of aperture 20 mm, the microscope field of view has a diameter of approximately 1 mm (20 mm/20). In this case, the volume of the field of view will be $V = h \cdot \pi r^2 = 0.01571$ mm^3 or about 15.7 nl, where h=20 µm is a depth of chamber. Calculating the spermatozoa in several fields of view it is possible to express sperm concentration in million/ml.

The precision of the evaluation of sperm concentration depends on the number of spermatozoa counted. The standard error (*SE*) of a count (*N*) can be conveniently expressed as a percentage of the count:

$$SE = \sqrt{N}.$$

The 95% confidence interval for the number of spermatozoa in the volume of semen is $N \pm 2\sqrt{N}$. By counting 1000 cells, the sampling error has been estimated to be around 3% which is acceptable for these experiments.

To evaluate semen with a concentration higher than 50 M/ml, 10 µl of semen sample was diluted in 90 µl of HEPES-buffered medium in order to adjust the concentration to a range that CASA can accurately evaluate (between 2 M/ml and 50 M/ml).

In the cases with a concentration lower than 2 M/ml (primarily the fractions recovered from the patients' semen samples), a FastRead 102 counting chamber and Olympus bright field microscope were used. The detailed information about FastRead 102 is presented in the Appendix 8.3.

2.2.2 Motility characteristics

Sperm motility parameters includes: straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), straightness (STR, or VSL/VAP) and linearity (LIN, or VSL/VCL). General motility classification included: rapid cells (VAP > 25 µm/s); moderate (VAP 5-25µm/s) and slow (VAP < 5µm/s and VSL < 11µm/s).

Progressive motility was classified as any cells with a VAP > 25μm/s and a straightness >80%.

Linearity (ratio of straight line velocity to curvilinear velocity) describes the percent of sperm moving in a straight line path:

$$Linearity = \frac{Straight\ line\ velocity\ (VSL)}{Curvilinear\ velocity\ (VCL)} \times 100$$

ALH is amplitude of lateral head displacement or a magnitude of lateral displacement of a sperm head about its spatial average trajectory. Figure 2.1 shows schematic representation of motility patterns measured by the CASA system. Black circles represent successive images of the head of a motile sperm and are joined by straight path lines. Curved line indicates a smoothed path fitted through sperm track.

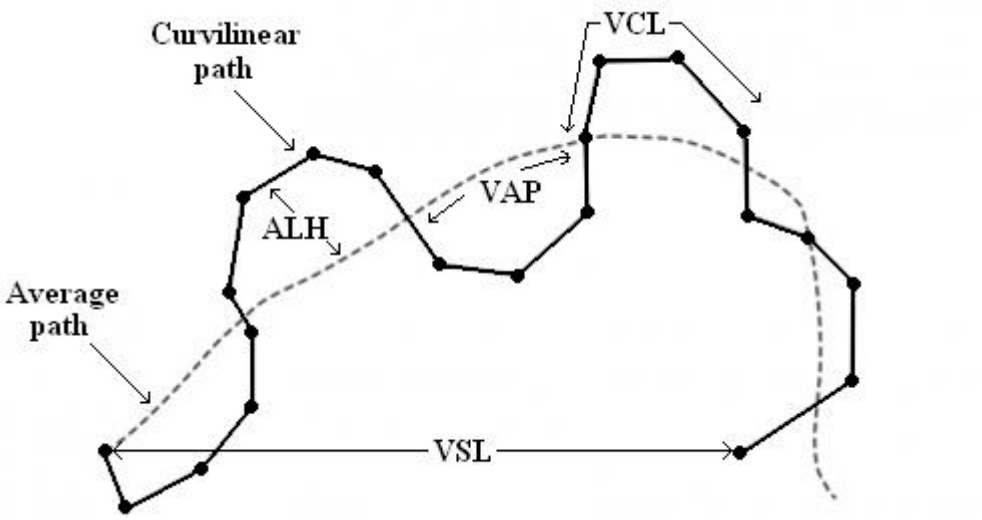


Figure 2.1 Diagram and the main motility parameters of sperm movement, including the velocity, the width of the sperm head trajectory and the frequency of the change in direction of the sperm head movement, measured by CASA (WHO, 2010).

2.2.3 Sperm morphology

The morphology of spermatozoa was analyzed using the Papanicolaou stain and Kruger criteria for normal spermatozoon morphology as described in the 5th edition of WHO laboratory manual for examination and processing of human semen (WHO, 2010). The Papanicolaou stain provides good staining of spermatozoa and allows excellent visualization of sperm defects (e.g. Fig.2.2). It stains the acrosomal and post-acrosomal regions of the head, excess residual cytoplasm, the midpiece and the principal piece.

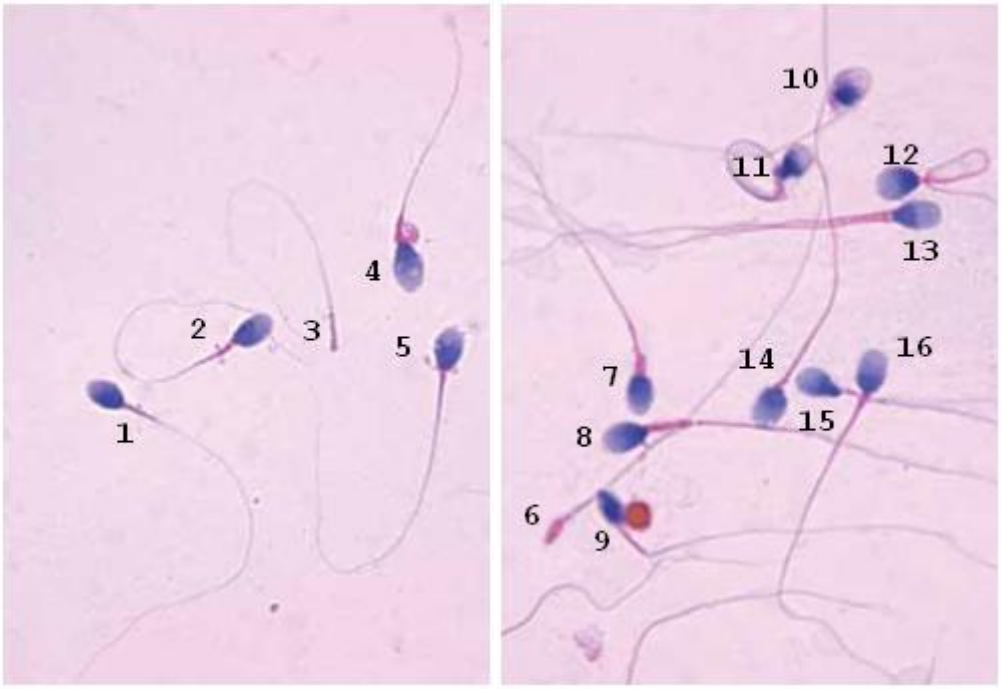


Figure 2.2 Spermatozoa stained with Papanicolaou (WHO, 2010). Sperm defects: 1 – normal; 2 – normal; 3 – pinhead; 4 – abnormal head with acrosome region >70%, thick midpiece and cytoplasmic droplet; 5 – normal; 6 – pinhead; 7 – abnormal midpiece; 8 – abnormal midpiece; 9 – abnormal head, bent principal piece; 10 – abnormal head; 11 – abnormal head, coiled principal piece; 12 – abnormal head, thick midpiece, coiled principal piece; 13 – double principal piece; 14 – normal; 15 – pyriform abnormal head; 16 – normal.

The main components of this method are: Harris's haematoxylin – to stain the nucleus, Orange G and Eosin Azure which are used for staining different regions of cytoplasm (Cell Path, UK). The full protocol of staining the sperm can be found in the Appendix 8.4.

Determination of sperm morphology comprises the following steps:

- Preparing a smear of semen or prepared fraction of spermatozoa on a slide.
- Air-drying, fixing with 95% ethanol and staining the slide.
- Examining the slide under Nikon S-Kt bright field microscopy at ×1000 magnification with oil immersion.
- Assessing approximately 200 spermatozoa per slide for the percentage of normal and abnormal forms.

Once the smears have been air-dried, they were fixed and stained to highlight details of the spermatozoa. In order to avoid the bias during the study of morphology, the donor number and a method of preparation (VMP or swim up) were written on the labeling area of the opposite side of a slide. The slides were mixed after preparation. The slide was turn up and the information about the number of donor and the method of preparation became available only after the examination was completely finished and the details about the defects were put down in a log book.

The percentages follow the binomial distribution, i.e. sperm are normal or abnormal. For this distribution, the standard error of the estimated percentage (p) within a class depends on the true, but unknown, percentage, as well as on the number of spermatozoa counted (N). The standard error is

$$SE = \sqrt{\frac{p(100-p)}{N}}$$

and an approximate confidence interval can be constructed from the normal distribution. If 200 spermatozoa are counted, and the percentage with normal morphology is 15% the standard error is 2.5%.

2.2.4 Measurement of Ca^{2+} response for progesterone

After isolation of spermatozoa from seminal plasma and capacitation for 3 hours in STF, the concentration was adjusted to 10 M/ml with STF. 1 µl of dye Fura2 (1µM) and 2.5 µl Pluronic F-127 (0.05% w/v), were loaded into 500 µl aliquot of sperm suspension in the dark (Nash *et al*., 2010). 45 min after incubation in 5% of CO_2, 37° C, the aliquot was centrifuged twice for 10 min at 500 g, supernatant was discarded and 45 µl of sperm suspension was placed into a 96 well plate.

Ca^{2+} response to progesterone was evaluated by the FLUOstar Omega (BMG Labtech Offenburg, Germany). This device measures fluorescence at 340 nm and 380 nm by highlighting the sample with UV light (510 nm). At 380 nm free molecules of Fura2 are the source of fluorescence. At 340 nm molecules of Fura2, which have bound to intracellular ions of Ca^{2+}, are the source of fluorescence. After the aliquots of 45µl of samples are loaded into a 96 well plate and 20 readings of control data (100 seconds) are acquired. Injecting 5 µl of 36 µM progesterone (final concentration is 3.6 µM) into the sample enhances the flow of Ca^{2+} into sperm. The concentration of free Fura2 drops and the signal at 380 nm

rapidly reduces. Therefore, the concentration of Fura2 which is bound with intracellular Ca^{2+} increases and that leads to amplification of the signal at 340 nm. During the time (~ 1 min) spermatozoa are saturated with Ca^{2+} and the signals reach a plateau. Injecting 5µl of 100 mM $MnCl_2$ absorbs Fura2 and, therefore, quenches the signals at 340 nm and 380 nm. The signals after injection of $MnCl_2$ provide the information about basal level of fluorescence in the sample. Thus, it is possible to determine the level of Ca^{2+} response for progesterone. Typical signals at 340 nm and 380 nm and their ratio are presented at the Figure 2.3.

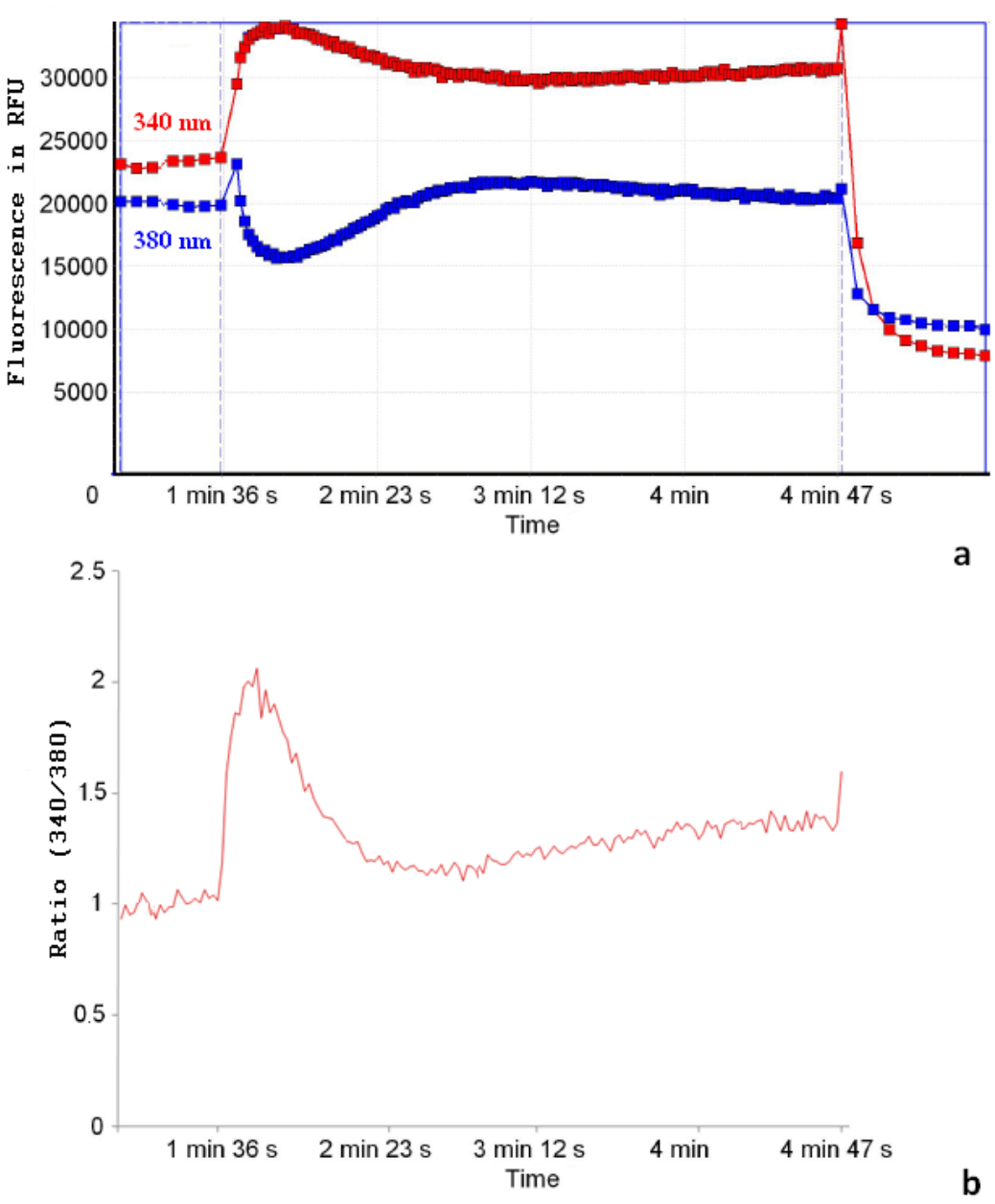


Figure 2.3 Typical fluorescence signals at 340 nm and 380 nm (a) and their ratio (b). An increase of 340 nm signal and decrease of 380 nm signal after injection of progesterone (at 1 min 36 s) shows the concentrations of dye bound with Ca^{2+} and concentration of free dye respectively. Signals drop to basal level after injection of $MnCl_2$ (4 min 45 s) which quenches the dye.

2.2.5 Hyaluronic acid binding

The hyaluronic binding ability of sperm was evaluated by the HBA® Sperm Hyaluronan Binding Assay, a dual chambered slide containing an attached layer of hyaluronan located beneath two individual cover slips (Biocoat, Inc., Horsham, PA). Following the manufacturer's instructions (Origio, Inc., 2011), the hyaluronic binding score was determined by dividing the number of motile spermatozoa bound with hyaluronic acid by the number of total motile sperm. Bound sperm are distinctly different from unbound in that they demonstrate rapidly beating tails with no movement while unbound sperm swim freely.

2.3 Media

Different types of media were tested (see Appendix 8.5) before choosing the basic medium for the VMP method. The basic medium used for all experiments was prepared according to the manufacturer's instructions and was based on synthetic human tubal fluid (STF; Irvine Scientific, USA), consisting of 97.8 mM NaCl, 4.69 mM KCl, 2.04 mM $CaCl_2$, 0.2 mM $MgSO_4 \cdot 7H_2O$, 2.78 mM D-Glucose, 0.33 mM Na pyruvate, 21.4 mM Na lactate, 25 mM $NaHCO_3$, and 30 mg/ml BSA. A non-capacitating HEPES-buffered medium (NCB) was adapted from STF without albumin and sodium bicarbonate with 25 mM HEPES (Sigma, UK). pH and osmolality of both media were adjusted to 7.4 and 290 – 300 mOsm/kg respectively.

2.4 Sperm preparation

2.4.1 Control and study groups

The ejaculate was split for two samples each of them was prepared by swim up and by the VMP method. Spermatozoa recovered by swim up formed a control group, spermatozoa recovered by the VMP method formed a study group.

2.4.2 Sperm preparation for control group – swim up

Swim up method was performed according the 5th edition of WHO laboratory manual for examination and processing of human semen (WHO, 2010). 5 ml of NCB was under laid with1 ml of semen, placed at angle of 45° to increase the surface of contact between semen and NCB and incubated in non CO_2 incubator at 37° C for 1 hour. After this incubation period, 2 ml of NCB was carefully aspirated from the top layer and placed into a sterile tube (Eppendorf, UK).

2.4.3 Sperm preparation for study group – novel spermatozoa selection method (viscous medium penetration)

The viscous medium penetration method was performed on raw semen. A line of 100 µl pre-warmed methylcellulose medium was drawn on a 35 mm plastic Petri dish (Corning, USA). A 100 µl drop of semen and 100 µl drop of STF medium were placed on the top and the bottom of this line respectively (Fig.2.4). Drops of semen and STF were carefully joined with the line of viscous medium. The large difference in viscosity of methylcellulose medium (~ 2000 cP) and STF (~ 1 cP) and surface tension of STF drop at the dish walls prevent dilution of viscous medium. The dish was incubated for 1 hour in a humidified atmosphere at 37°C

with 5% of CO_2. After this period of time, a drop of STF was aspirated and placed into a sterile tube (Eppendorf, UK).

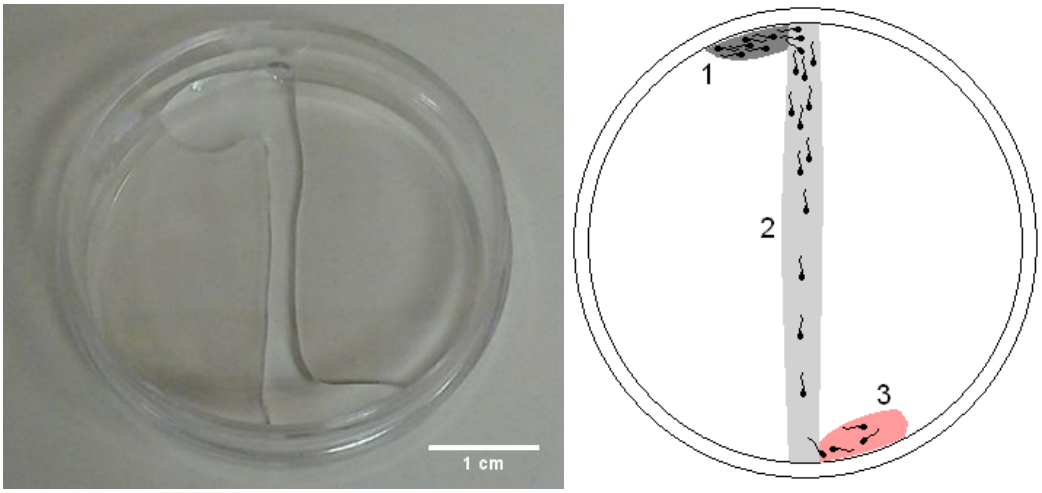


Figure 2.4 Photo and principle of the VMP method illustrating sperm penetration through the line of viscous medium. 1 – a drop of semen, 2 – a line of viscous medium, 3 – a drop of STF for sperm pick-up.

2.5 Repeatability and reliability of the technique

The repeatability of the sperm selection technique was established by splitting the portion of semen sample into 5 equal aliquots. Each aliquot was processed in a separate dish by the VMP method. Samples varied in concentration, motility and morphology of spermatozoa that allowed testing the technique for different quality of semen.

One of the important aims of these experiments was to determine the reliability of sperm selection technique. The approach was to take different aliquots of the same semen sample and to apply swim up and the VMP method. Spermatozoa

recovered by these procedures were studied and their characteristics were compared.

2.6 Statistical analysis

Microsoft Office Excel 2007, Advanced Grapher and Sigma Plot were used to process the data and plot the graphs. Results are expressed as the mean ± standard deviation, mean ± standard error, average value or range. Statistical differences between the values were made using analysis of variance (ANOVA) if the data were originally normally distributed. If data were not normally distributed the statistical difference was determined by Mann-Whitney rank sum test. The correlations between sperm characteristics and penetration ability were examined using either Pearson's correlation or Spearman's correlation coefficient, depending on the normality distribution of the data. Differences with $P < 0.05$ were considered as significant.

Chapter 3 Development of the Viscous Medium Penetration Method

3.1 Introduction

The viscous medium penetration (VMP) method is based on the hypothesis that sperm that have passed through the cervical mucus have better fertilization ability than the sperm in the ejaculate. Previously, this fact was observed in the experiments with animal cells (Cohen & McNaughton, 1974; Fischer & Adams, 1981; Siddiquey & Cohen, 1982).

However, there are no published studies reporting similar research with human gametes. Due to technical and ethical limitations in obtaining spermatozoa from the female reproductive tract and fertilizing the oocytes with recovered sperm, it is extremely difficult to carry out such a project. However, it is possible to develop a system to simulate the process of sperm selection that may take place *in vivo*. Kremer offered a system for assessing sperm penetration by filling a capillary tube with mid-cycle cervical mucus (Kremer, 1964). Sperm penetration of mucus is examined by evaluation of sperm concentration and motility at different distances of the tube. Several clinical studies have reported that sperm penetration through cervical mucus *in vitro* correlates with fertilization rates *in vivo* and *in vitro* (Aitken *et al*., 1992; Barratt *et al*., 1989).

Despite these promising results, no technique has yet been introduced for recovering the penetrated sperm. The VMP method is the first reported attempt to develop and test this system. The efficiency of the system depends on several factors such as design, media, and processing conditions. This chapter is devoted to the investigation of these aspects.

3.2 Design of the viscous penetration method

In order to find the optimum and the most robust configuration, different modifications of the viscous medium penetration (VMP) method were tested (Fig. 3.1).

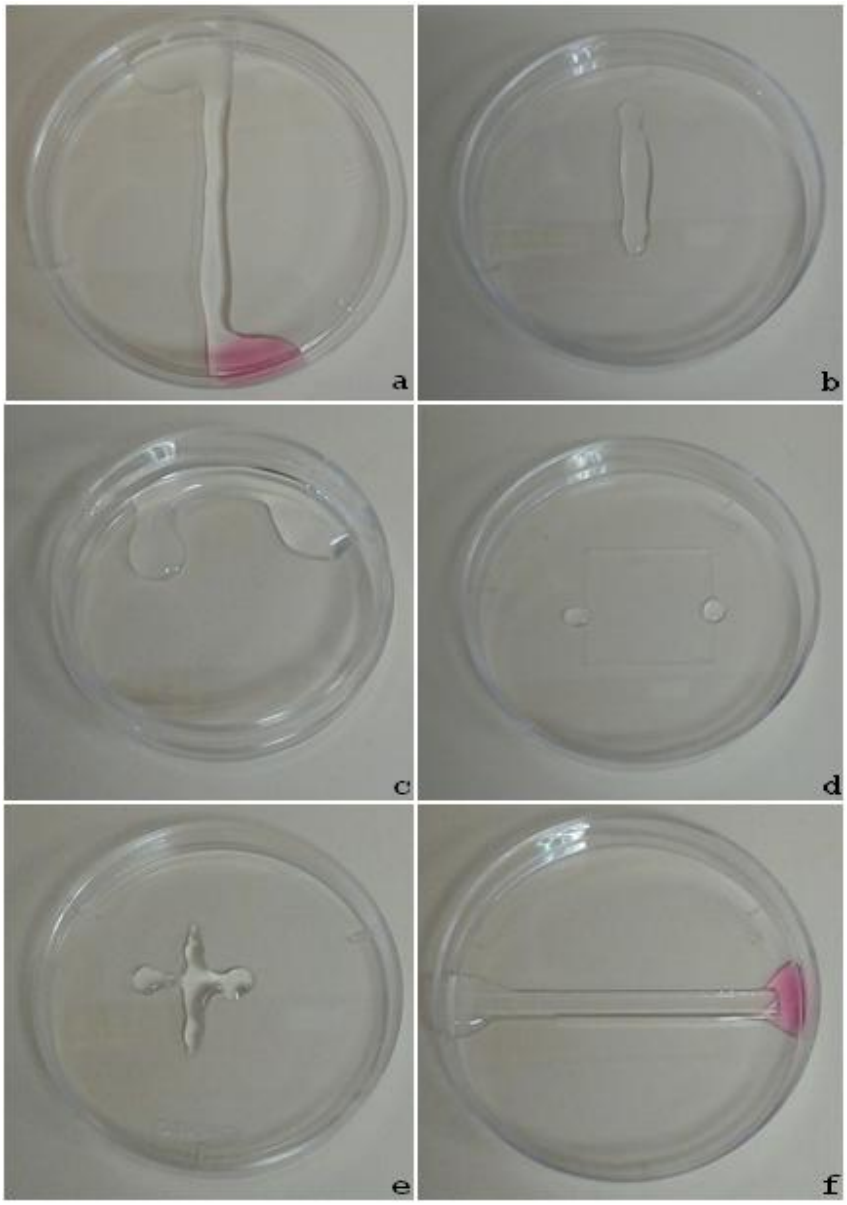


Figure 3.1 Different dish modifications tested in order to find the optimum configuration. The major disadvantages: a – the viscous line is too long; b – the viscous line is too short; c – inappropriate way of solutions connection; d – usage of cover slip which makes the viscous area is too wide and makes hard to recover sperm; e – multiply way of sperm journey; f – usage of capillary tube which makes impossible the sperm recovery.

The size of the tested dishes varied from 35 mm to 70 mm. In order to minimize the mixing of semen, viscous solution and synthetic tubal fluid (STF) it was necessary to place the drops near the walls of the dish. Surface tension and a great difference in the viscosities slow down the diffusion in the solutions and prevent the sperm displacement by the flows of media.

A large number of versions of the VMP method was tested. In early versions of the VMP method, cover glass or glass tubes were used in order to minimize the evaporation of water from the media and prevent drying of semen. This approach was technically more difficult and not effective. It required extra materials and an additional step of loading a glass tube with viscous solution. Also, it was difficult to recover spermatozoa from the glass tube or from the drop which was placed under the cover slip.

The site where a drop of semen is connected with the line of viscous medium was chosen according to the average width of external os of cervix – 5 mm (Wright *et al*., 2002).

The length of the viscous line is one of the most important parameters in the VMP method. If the line is too short, less than 2 cm, the yield contains poor motile sperm and cell debris, which must not be present in the prepared fraction. With a too long line, more than 5 cm, it is impossible to recover sufficient number of spermatozoa that could be used in IVF. In the final configuration of the dish the length of the line was chosen according to the average length of female cervix in the middle of menstrual cycle – 35 mm (Fig.3.2).

As the volume of cervical mucus plays a significant role in sperm transport (Elstein, 1978), the volume of viscous medium in the VMP method can influence the number of spermatozoa that will be recovered. *In vivo*, an average volume of cervical mucus in the middle of menstrual cycle is approximately 3 ml (Elstein, 1978; Murakov *et al*., 2009) which is close to the average volume of semen ejaculated into vagina. Taking into account this factor and the preliminary experiments it was decided to use the same volumes of semen, viscous solution and STF medium. In order to recover more sperm from the samples it is possible to split the semen sample and prepare several dishes. Thus, the total number of recovered sperm will be equal: $N = 0.1 \times n \times C$, where n – the number of prepared dishes, C– the concentration of sperm in the recovered fraction.

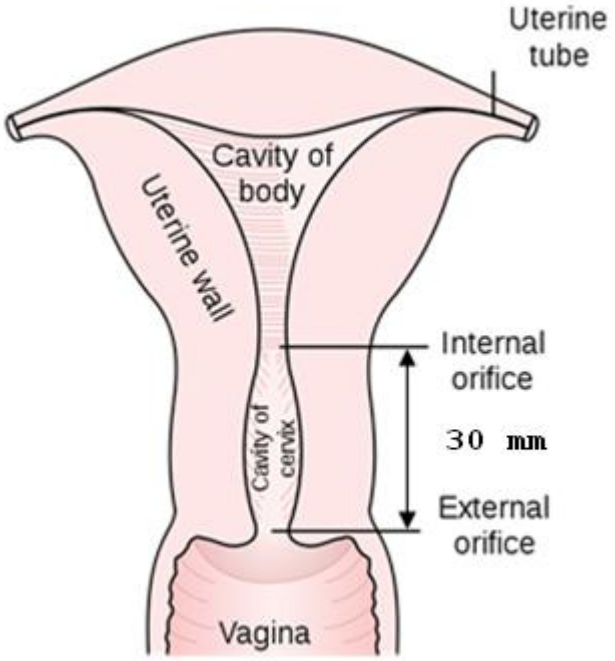


Figure 3.2 Female reproductive tract with vagina, cervix and uterus (Gray, 1989). The size of the cervix is similar to the length of viscous medium.

3.3 Viscous solution

Mid-cycle human cervical mucus is difficult to obtain in large quantities and it degrades quickly after collection (Katz, 1991). The characteristics of cervical mucus can be also affected by the drugs usually employed for induction of superovulation (Gelety & Buyalos, 1993; Randall & Templeton, 1991). Therefore, mucus is not a reliable component for the VMP method. For this reason, a synthetic substitute for cervical mucus was used. Cervical mucus is composed of mucus glycoproteins, proteins (e.g., lactoferrin), enzymes, amino acids, cholesterol, lipids, and a range of inorganic ions, the concentrations of which fluctuate during the menstrual cycle (Marriott *et al.*, 1990). Water makes up between 90% and 99% of mucus (Katz & Dunmire, 1993). Mid-cycle cervical mucus from a non pregnant premenopausal human has a pH of approximately 7.4 (Tang *et al.*, 1999). The primary biochemical component of mucus is the mucin glycoprotein, which is believed to be largely responsible for the viscous nature of mucus. Dried porcine gastric mucin is available commercially, but it does not regain its original viscosity upon rehydration (Kočevar-Nared *et al.*, 1997). Clift *et al.* (1953) determined that the range of viscosity of cervical mucus in the middle of menstrual cycle was between 1500 and 2500 cP (1 cP = 1 mPa·s). Ishijima *et al.* (1986) report a range between 2500 to 6500 cP, and Karni *et al.* (1971) who studied 200 women reported a viscosity of 500 to 3000 cP for mid-cycle human cervical mucus. Consequently, a target viscosity of 2000 cP for a synthetic cervical mucus formulation was chosen. Methylcellulose (MC) is the most effective medium as a model of cervical mucus (Barratt *et al.*, 2001; Ivic *et al.*, 2002), because it is non-toxic, has standardized viscosity (10 – 10000 cP),

commercially available, inexpensive and solutions are stable over long period of time. Thus, in these experiments viscous medium was prepared by dilution of methylcellulose (Sigma, UK) in STF.

3.4 Environment conditions

Conditions in the dish are extremely important for proper processing semen by the VMP method. To make the conditions close to conditions in female reproductive tract, it was decided to incubate the dish in a humidified atmosphere at 37°C with 5% of CO_2. This environment minimizes evaporation of media and maintains viscosity, pH and concentrations of chemical components stable. Evaporation of water from the solutions significantly influences the ability of spermatozoa to penetrate the viscous medium. Evaporation changes the viscosity, concentrations of chemical components and osmolality in the solutions. To study the effect of evaporation, drops of 100 µl STF each were placed on a Petri dish and incubated for 60 minutes in 5% CO_2 incubator with 95% humidity. After that each drop was aspirated and the volume was measured. On average, the volume loss was 6 µl that led to an increase the osmolality of 4 mOsm. This increase does not affect sperm motility (Rossato *et al.*, 2002). With non CO_2 incubator and HEPES-buffered medium the evaporation was significantly higher. On average, the volume of a drop decreased by 30% during 1 hour of incubation. Therefore, the final osmolality of this solution was 350 mOsm, which is out of physiological range of osmolality of fluids in the female genital tract (280-300 mOsm) (Fisch *et al.*, 1990). The lack of $NaHCO_3$ can also inhibit sperm motility and affect penetration ability of sperm (Boatman & Robbins, 1991). These facts were confirmed experimentally. It was found that with the non CO_2 incubator the yield

of the VMP method was very low. Despite high concentration and good motility of sperm in semen, only few spermatozoa had a capacity to penetrate through viscous medium in these conditions which is not enough for IVF (Fig. 3.3).

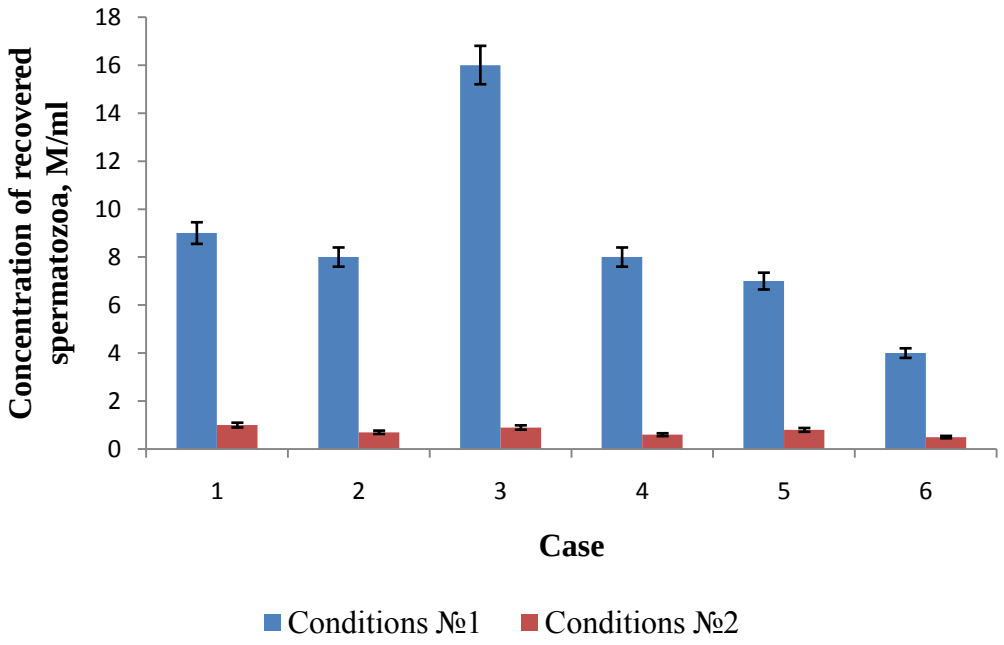


Figure 3.3 Concentration of recovered spermatozoa by the VMP method performed under different incubation conditions. Each vertical line indicates ± standard deviation of individual readings about the mean. Conditions №1: STF, 60 min in 5% of CO_2, 37° C and humidity 95%. Conditions №2: HEPES-buffered medium, 60 min at 37° C. The number of recovered cells was significantly lower ($P<0.05$) if the VMP method was performed under conditions №2.

3.5 Time of incubation

Time of incubation in the VMP method is significantly important for the concentration and motility of recovered spermatozoa. The relationships between the concentration of sperm recovered by the VMP method and the time of incubation are presented at Figure 3.4. The relationship was examined for the

normozoospermic samples and for the samples obtained from the patients with oligoasthenoteratozoospermia, when the concentration, motility and morphology of spermatozoa in semen were below the WHO criteria. The statistically significant difference in the concentrations was observed only between 30 and 60 minutes of incubation irrespective of the initial concentration of sperm in the sample. For samples obtained from the normozoospermic donors, there was on average a 53% increase in concentration of sperm after 60 min of incubation in comparison with 30 min of incubation, a 7% increase after 90 min of incubation and a 3% increase after 120 minutes of incubation. For the oligoasthenteratoozoospermic samples, there was a 50% increase in concentration of spermatozoa after 60 min of incubation in comparison with 30 min of incubation, a 20% increase after 90 min of incubation and a 9% increase after 120 minutes of incubation.

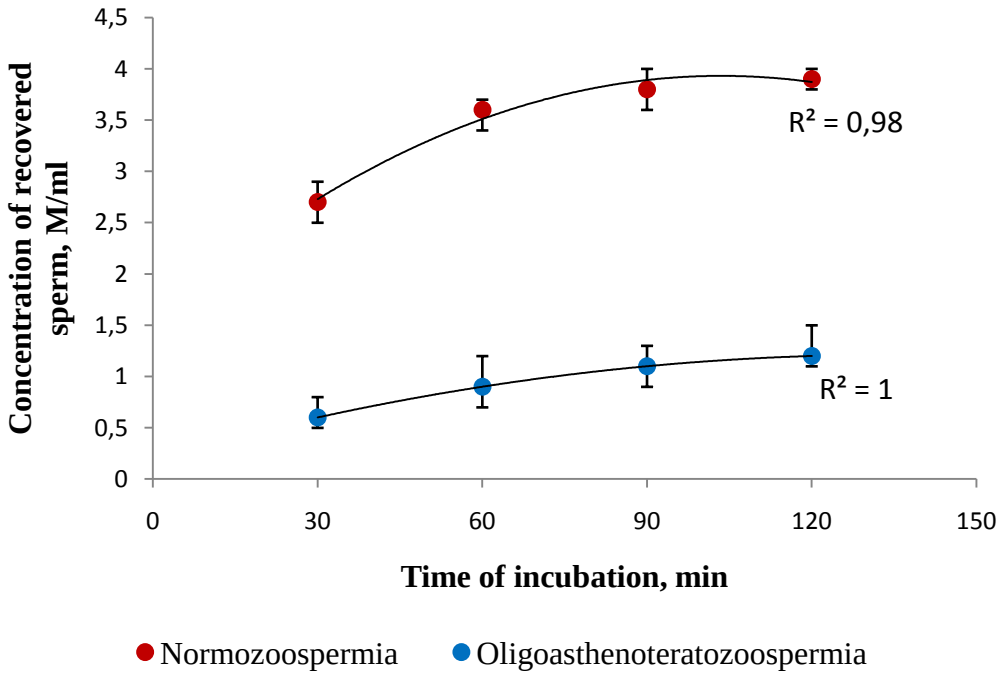


Figure 3.4 Relationship between the concentration of recovered spermatozoa from normozoospermic and oligoasthenoteratozoosperic samples and the time of

incubation of the dish. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from donors' and patients' samples (n=6). The number of recovered cells is significantly higher (P<0.05) between 30 and 60 minutes of incubation. There was no statistically significant difference (P=0.3) in the number of recovered cells between 30 and 120 minutes.

Despite the increasing concentration of sperm, a decrease in percentage of total motile spermatozoa with the time of incubation was observed. The relationship between the percentage of total motile sperm recovered by the VMP method and time of processing semen, i.e. time of incubation of the dish is presented at Figure 3.5. Although, there was no statistically significant difference between the neighboring values (P=0.4), a statistically significant difference between the total motility of sperm at 30 and 120 minutes was found (P<0.05).

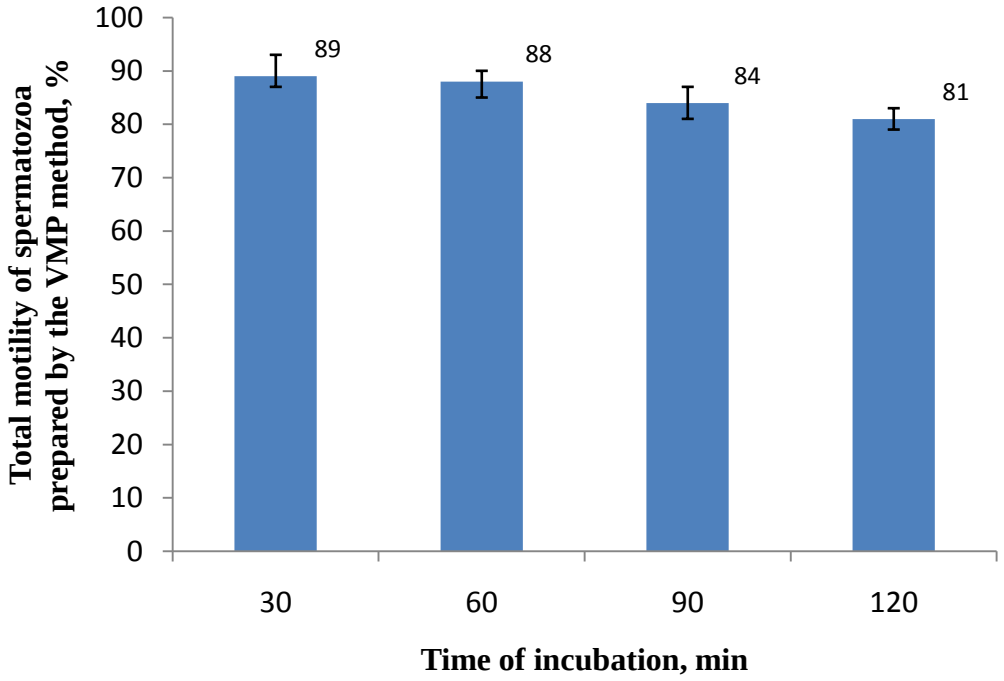


Figure 3.5 Relationship between the percentage of total motile spermatozoa and the time of incubation of the dish. Each vertical line indicates ± standard deviation for total percent motile sperm recovered by the VMP from donors' samples (n=3).

There was no statistically significant difference ($P = 0.4$) between the neighboring values. There was a statistically significant difference ($P < 0.05$) between the total motility of sperm at 30 and 120 minutes.

Thus, the time for processing semen by the VMP method was chosen equal to 60 minutes. This period is the most optimal balance between concentration of recovered sperm and their motility. Also, it is equal to the time of preparation spermatozoa by swim up, which makes the results more comparable.

3.6 Discussion

The VMP method was developed in order to build a model of sperm selection comparable to the female cervix. The principal difference from the penetration test is the ability to recover sperm passed through the viscous medium. This gives the opportunity to examine sperm characteristics that have not been previously accessible. These characteristics include sperm motility patterns, morphology, functional capacity. Furthermore, recovered sperm can be potentially used in IVF.

Currently, this technique is a complex system with many variable parameters which can influence the efficiency of sperm recovery. However, after this method was adjusted, the experimental protocol of sperm isolation became easy and robust. All the components of the system were created with the commercially available compounds and chemicals. This approach to development of the VMP technique makes the system universal and easily repeatable in other laboratories. The only component that has to be specially prepared is a viscous solution. However, methylcellulose, which formed the basis of the viscous solution, is well

known and widely used in medicine, pharmaceuticals and other areas related to healthcare. Although it is not routinely used in IVF, methylcellulose is safe and can be used in the solutions for human cells.

As no complex equipment or expensive media are required for processing semen by this novel method, the system is relatively cheap and easy to use. Although the time of processing semen by the VMP is greater than the time of DGC, the VMP technique requires fewer manipulations from the embryologist to perform it.

Although this system is not designed for processing the whole ejaculate, the preliminary experiments have demonstrated that it is possible to recover adequate amount of sperm for IVF.

The ability of the VMP method to isolate sperm from semen and the quantitative characteristics of these spermatozoa will be described in the next chapter.

Chapter 4 Quantitative Characteristics of Spermatozoa Recovered by the Viscous Medium Penetration Method

4.1 Introduction

Sperm concentration and motility are the central characteristics of male fertility as they determine the migration in the female reproductive tract (Katz *et al.,* 1989). Males with low number of spermatozoa and/or poor motility are subfertile (Guzick *et al.,* 2001). However, information about concentration and motility is not robust enough to predict fertilization and resulting pregnancy especially in IVF (Bongso *et al*., 1989; Donnelly *et al*., 1998). Moreover, traditionally, sperm concentration and motility are assessed by eye when looking through a microscope, but this approach is subjective (Mortimer *et al.,* 1986).

In order to overcome the variability, computer assisted semen analysis (CASA) was developed (Dott & Foster, 1979). CASA carries out an objective and accurate study of sperm concentration and motility. Some authors report that, although the difference in sperm concentration evaluated by visual observation or by CASA was relatively small, the analysis of sperm motility was more accurate when CASA was used (Garrett *et al.,* 2003; Tomlinson *et al.,* 2010). CASA has been demonstrated to be a useful tool to assess kinematic properties of each individual spermatozoon in an ejaculate or prepared fraction (Larsen *et al.,* 2000; Mortimer, 2000). Extensive research has addressed the issue of CASA sperm characteristics as predictors of male fertility (Aitken *et al*., 1984; Barratt *et al*., 1993; Hirano *et al.,* 2001). It has been shown that CASA has potentially greater value in predicting male fertility than the routine semen examinations (Suzuki *et al.,* 2002). Using CASA, it was found that the motility parameters are associated with the male fertility. Barratt *et al.* (1993) showed that the total number of spermatozoa and VAP can be the indicators of the chance of pregnancy. Irvine *et*

al. (1994) found that percentage of progressively motile spermatozoa as being significant covariates in the prediction of pregnancy. Other motility patterns: ALH, VSL, VCL have also been reported to be positively correlated with fertility (Aitken *et al*., 1984; Barratt *et al*., 1993; Irvine *et al*., 1994; Krause, 1995; Paston *et al*., 1994).

Thus, CASA is a powerful tool for objective analysis of sperm characteristics that may impact the fertilization ability. This instrument may be used to evaluate and compare the concentration and motility of spermatozoa recovered by swim up and the viscous medium penetration (VMP) method.

4.2 Aims and experimental design

The first aim of this chapter is to report the results of testing a novel method of selection spermatozoa based on viscous medium penetration. The second aim of this chapter is evaluate the parameters of sperm that are significant for the penetration through the line of viscous medium. The third aim is to compare the characteristics of sperm penetrated through viscous medium with sperm from unprepared semen and spermatozoa recovered after swim up.

To compare the experimental sperm preparation technique with a method in common clinical practice, each semen sample was divided into two groups. One group was designated the control, and prepared by swim up. The other group was exposed to the experimental processing – the VMP method. Measurements of sperm concentration and motility characteristics were made in all groups. The data

were analyzed by direct comparison of the relative values of control and study groups.

4.3 Materials and methods

Concentrations of recovered spermatozoa prepared by the VMP method and by swim up were compared with the initial concentration of spermatozoa in unprepared semen. 23 semen samples were examined from 23 different normozoospermic donors. Concentration of spermatozoa in semen varied from 35.1 M/ml to 224 M/ml with an average value 115 M/ml.

For the experimental preparation group, a 500 µl portion of semen sample was split into 5 dishes prepared as described in 2.4.3. The drops of synthetic human tubal fluid (STF) were carefully aspirated from the dish after 1 hour of incubation at 5% of CO_2, 37° C, humidity 95% and placed into a sterile tube. Control group was prepared by swim up as described 2.4.2 and placed into another sterile tube.

To calculate the penetration ability of sperm cells in the VMP method, it is necessary to divide total number of cells recovered after processing by the number of cells in the unprepared sample. For example, the initial concentration of sperm in semen was 224 M/ml. 500 µl of semen was divided into 5 dishes which equates to 112M cells in total (5x22.4 M = 112 M). After 60 minutes incubation, 100 µl of prepared fraction were recovered from each dish (500 µl total). The concentration of spermatozoa in this fraction was 20.2 M/ml, yielding 10.1 M. Thus, penetration ability: 10.1M/112M = 0.09 or 9%.

Motility characteristics of spermatozoa which were recovered by the VMP method were studied in this series of experiments and compared with the characteristics of spermatozoa in semen and spermatozoa recovered by swim up.

4.4 Results

4.4.1 Concentration of recovered spermatozoa

In the VMP method the average percentage of spermatozoa migrated from the drop of semen to the drop of STF was 9.2%. In swim up, 31.6% of sperm in semen migrated to the upper layer of the medium, which significantly higher ($P<0.05$) than in the VMP method. The concentration of spermatozoa after the VMP correlated well ($r = 0.92$) with the initial concentration of sperm in semen. Correlation between the concentrations in semen and after swim up was 0.96. The relationship between the concentrations of spermatozoa in semen and after VMP, relationship between the concentrations of spermatozoa in semen and swim up and the approximations are presented at Figure 4.1.

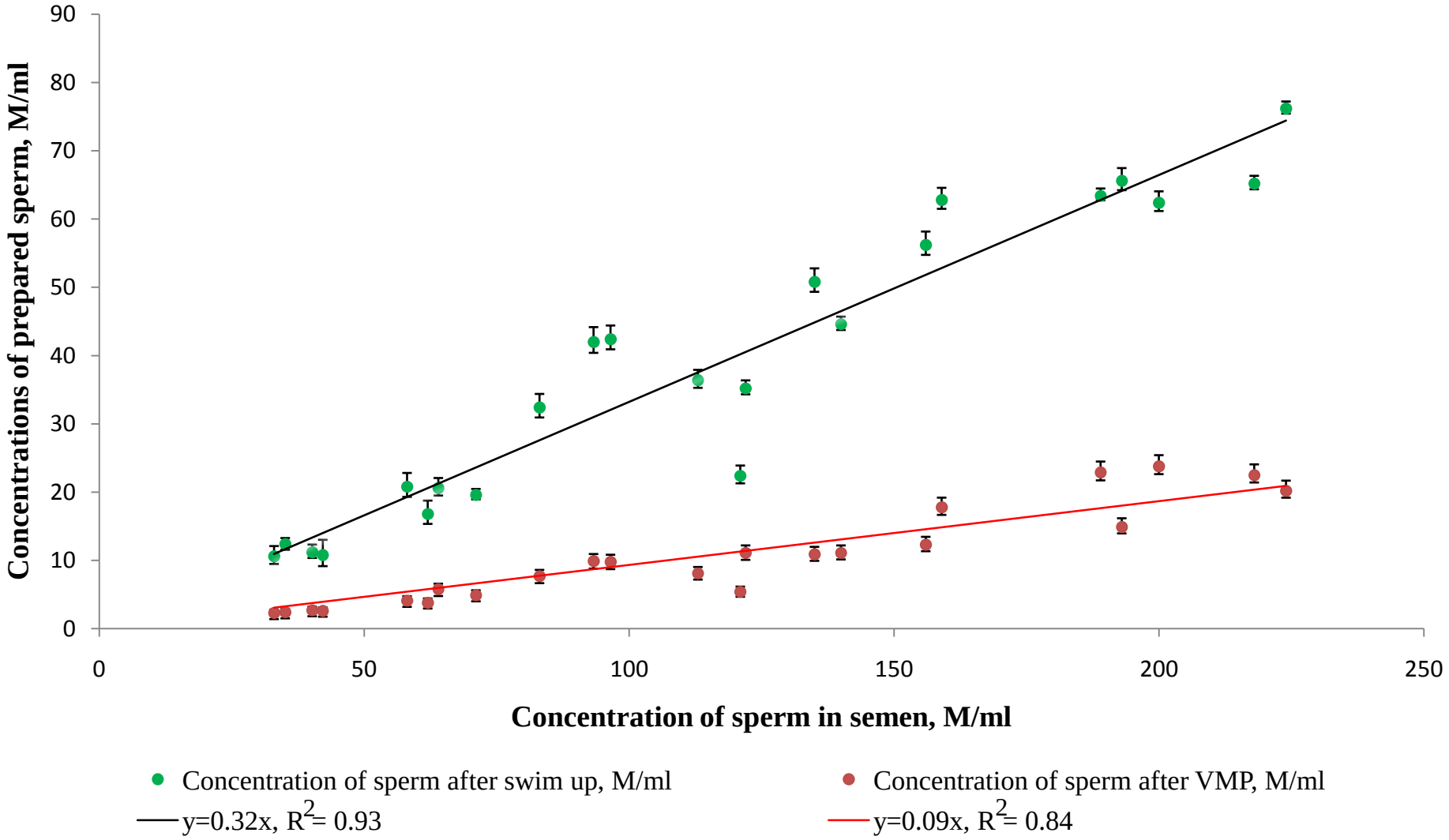


Figure 4.1 Relationships between the concentrations of spermatozoa in semen and spermatozoa recovered by the VMP method and swim up (number of semen samples: n=23). Each vertical line indicates ± standard deviation of individual readings about the mean. The difference between the methods of preparation is statistically significant ($P<0.05$).

4.4.2 Motility characteristics

4.4.2.1 Total motile spermatozoa

The Figure 4.2 shows the distribution of percentages of total motile spermatozoa in semen, spermatozoa that penetrated viscous medium and after swim up for all cases.

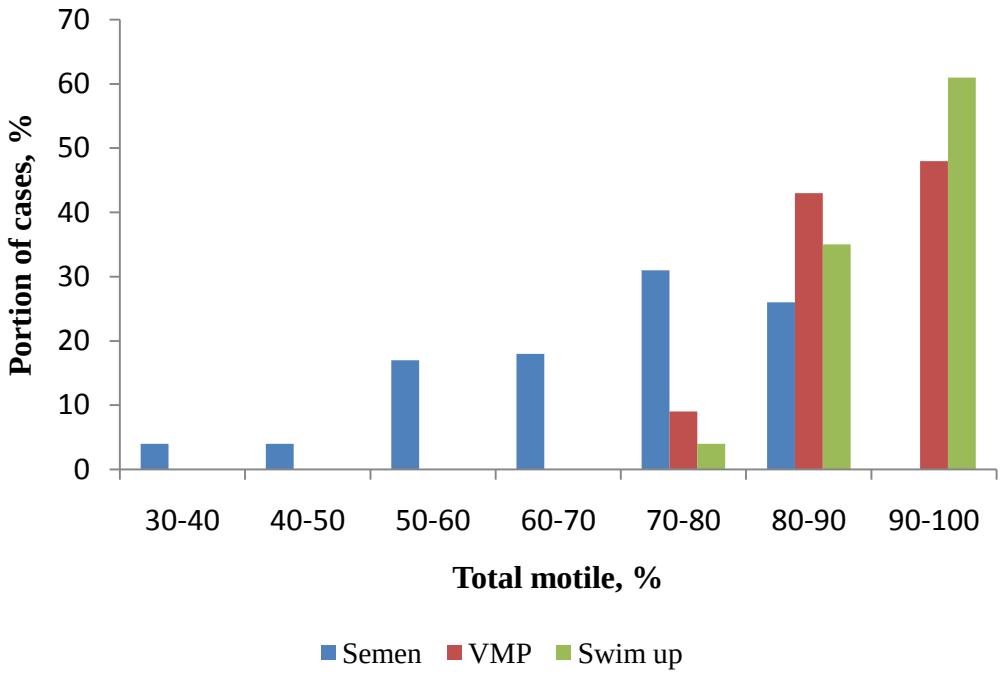


Figure 4.2 Distribution of percentages of total motile spermatozoa in semen, spermatozoa recovered by the VMP method (n=23). The portion of the cases with high percentage of total motile spermatozoa recovered by the VMP method and swim up are significantly higher ($P<0.05$) than in semen. The difference between the methods of preparation is not statistically significant ($P=0.15$).

The percentage of motile spermatozoa in semen varied widely from 38% to 88%, with an average value of 69%. After the preparation of semen, the percentage of motile sperm was significantly ($P<0.05$) higher, as would be expected. In all cases, the percentage of motile sperm in prepared samples was higher than 75%, independent of the preparation method used. Average percentage of motile sperm

was 88% after the VMP method and 90% after swim up. There was no statistically significant difference (P=0.15) in percent of total motile sperm between the two methods.

4.4.2.2 Progressive motile spermatozoa

The Figure 4.3 shows the distribution of percentages of progressive motile spermatozoa in semen, spermatozoa that penetrated viscous medium and after swim up.

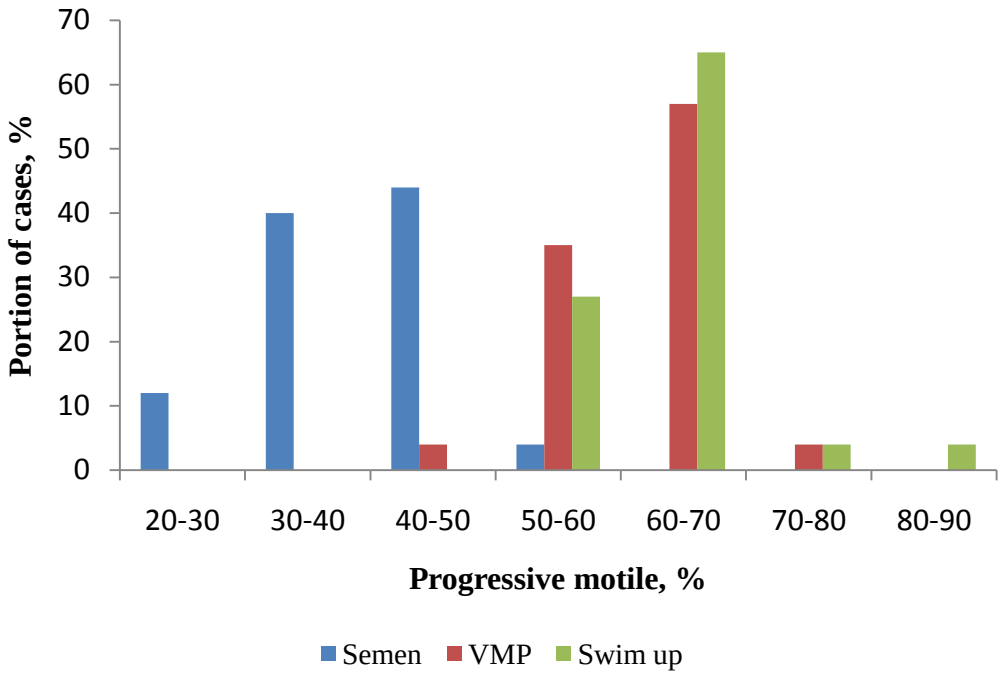


Figure 4.3 Distribution of percentage of progressive motile spermatozoa in semen, spermatozoa that penetrated viscous medium and swim up (n=23). The portion of the cases with high percentage of progressive motile spermatozoa after preparation by VMP and swim up are significantly higher (P<0.05) than spermatozoa in semen. The difference between the methods of preparation is not statistically significant (P=0.16).

The percentage of progressive motile spermatozoa in unprepared semen was also variable (24% – 54%) with an average value of 38%. After viscous medium penetration and swim up the percentage of progressive motile sperm was significantly ($P<0.05$) higher. The percentage of progressive motile spermatozoa recovered by the VMP method varied from 49% to 77%with an average value of 61%. After swim up the percentage of progressive motile spermatozoa varied from 51% to 82%, and the average percentage progressive motility was 64%. There was no statistically significant difference ($P=0.16$) in percent of progressive motile sperm between the two methods.

4.4.2.3 Straight line velocity

The Figure 4.4 shows the distribution of straight line velocity of spermatozoa in semen, spermatozoa that penetrated viscous medium and after swim up for all cases. Average VSL of spermatozoa from the semen samples was distributed between 20 μm/s and 60 μm/s, with an average VSL of 42 μm/s. Prepared spermatozoa had significantly higher VSL: average VSL after preparation by swim up and viscous medium penetration was distributed between 30 μm/s and 90 μm/s. Average VSL of spermatozoa after VMP of distribution was 55 μm/s, average VSL of spermatozoa after swim up of distribution was 53 μm/s. There was no statistically significant difference ($P=0.5$) in VSL between the two methods.

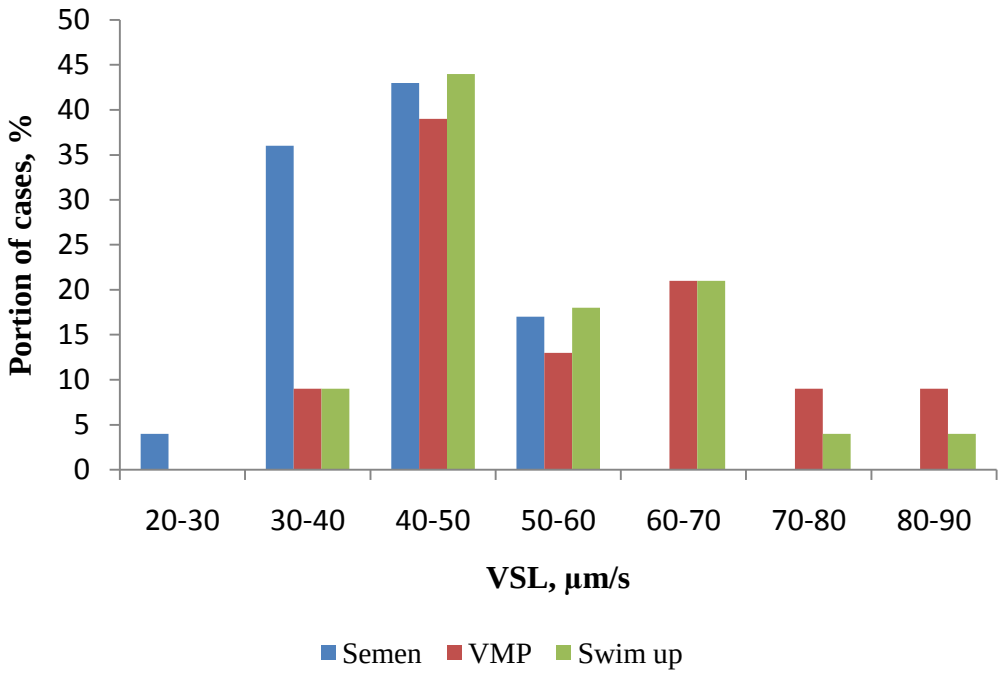


Figure 4.4 Distribution of average straight line velocity of spermatozoa in semen, spermatozoa recovered by the VMP method and swim up (n=23). The portion of cases when spermatozoa had high straight line velocity after preparation by VMP and swim up are significantly higher ($P<0.05$) than spermatozoa in semen. The difference between the methods of preparation is not statistically significant ($P=0.5$).

4.4.2.4 Curvilinear velocity

The Figure 4.5 shows the distribution of curvilinear velocity of spermatozoa in semen, spermatozoa that prepared by the VMP method and by swim up. Average VCL of spermatozoa in unprepared samples was distributed between 40 µm/s and 100 µm/s, with an average value of 75 µm/s. Prepared spermatozoa had significantly higher VCL: average VCL after preparation by swim up and viscous medium penetration was distributed between 60 µm/s and 160 µm/s.

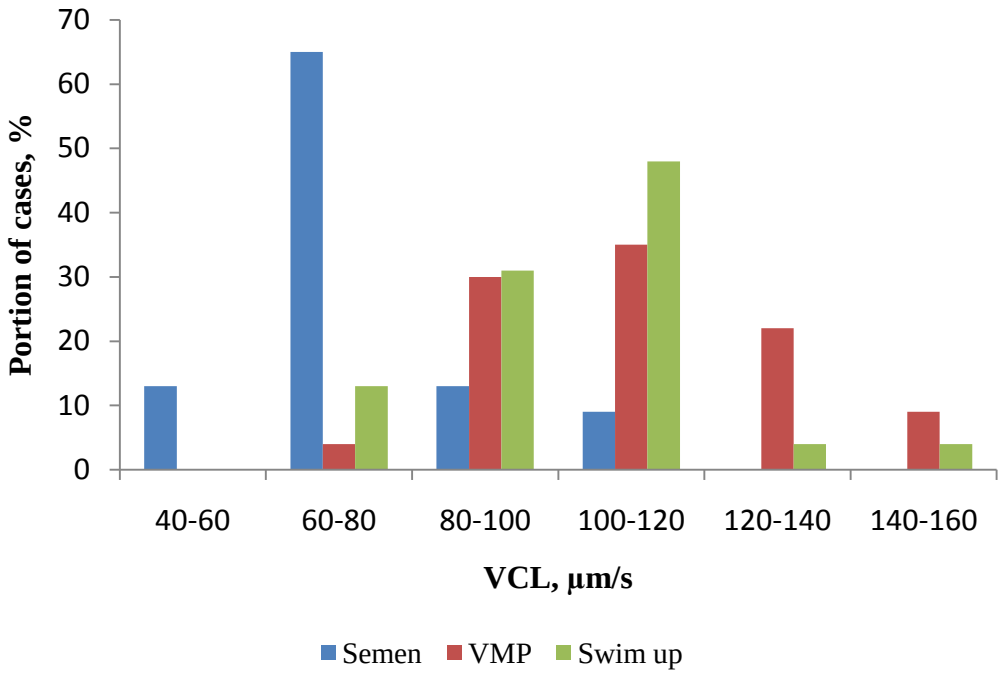


Figure 4.5 Distribution of average curvilinear velocity of spermatozoa in semen, spermatozoa recovered by the VMP method and swim up (n=23). The portion of cases when spermatozoa had higher curvilinear velocity after preparation is significantly higher (P<0.05) than spermatozoa in semen. The difference between the methods of preparation is not statistically significant (P=0.26).

Average VCL of spermatozoa recovered by the VMP method of the distribution was 108 µm/s, average VCL of spermatozoa after swim up of the distribution was 101 µm/s. There was no statistically significant difference (P=0.26) in VCL of sperm recovered by the both methods.

4.4.2.5 Average path velocity

The Figure 4.6 shows the distribution of average path velocity of spermatozoa in semen, spermatozoa recovered by the VMP method and swim up.

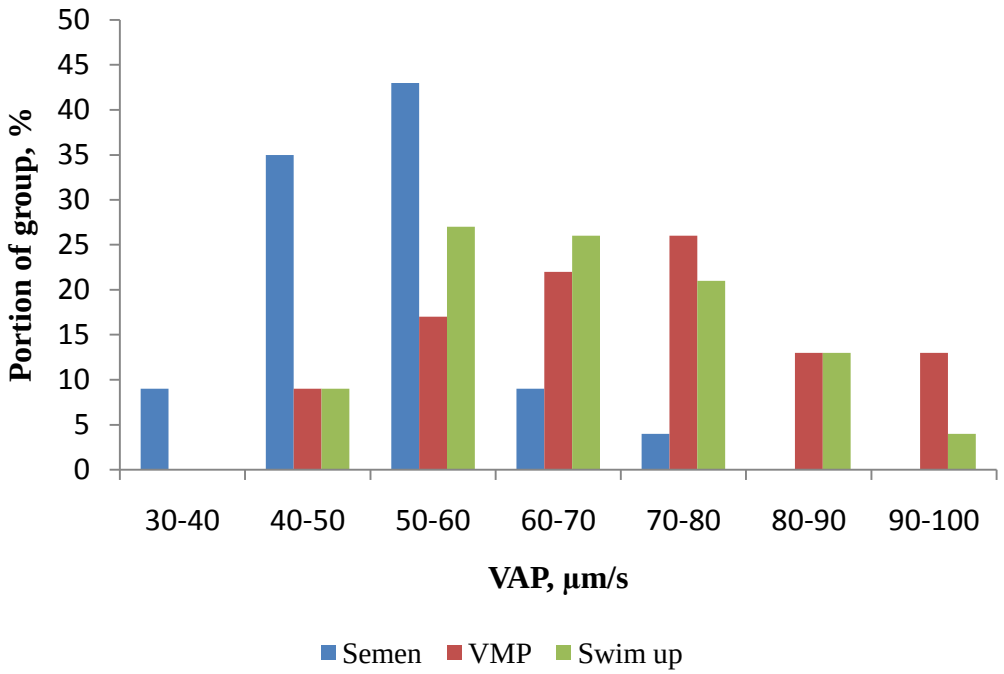


Figure 4.6 Distribution of average values of average path velocity of spermatozoa in semen, spermatozoa recovered by the VMP method and swim up (n=23). The portion of cases when spermatozoa had higher average path velocity after preparation is significantly higher ($P<0.05$) than spermatozoa in semen. The difference between the methods of preparation is not statistically significant ($P=0.36$).

Average VAP of spermatozoa from the semen samples was distributed between 30 µm/s and 80 µm/s, with an average VAP of 52 µm/s. Prepared spermatozoa had significantly higher VAP: average VAP after preparation by swim up and viscous medium penetration was distributed between 40 µm/s and 100 µm/s. Average VAP of spermatozoa after VMP of distribution was 71 µm/s, average VAP of spermatozoa after swim up of distribution was 67 µm/s. There was no statistically significant difference ($P=0.36$) in VAP between the two methods.

4.4.2.6 Amplitude of lateral head displacement

The Figure 4.7 shows the distribution of average amplitude of lateral head displacement of spermatozoa in semen, spermatozoa that recovered by the VMP method and swim up for all cases.

Average ALH of spermatozoa from unprepared samples was distributed between 2.5 µm and 4.5 µm, with an average value of 3.5 µm. Prepared spermatozoa had significantly higher ALH and there was a statistically significant difference between the methods of preparation: average ALH after preparation by swim up was distributed between 3 µm and 5.5 µm and average ALH after the VMP method was distributed between 3.5 µm and 6 µm.

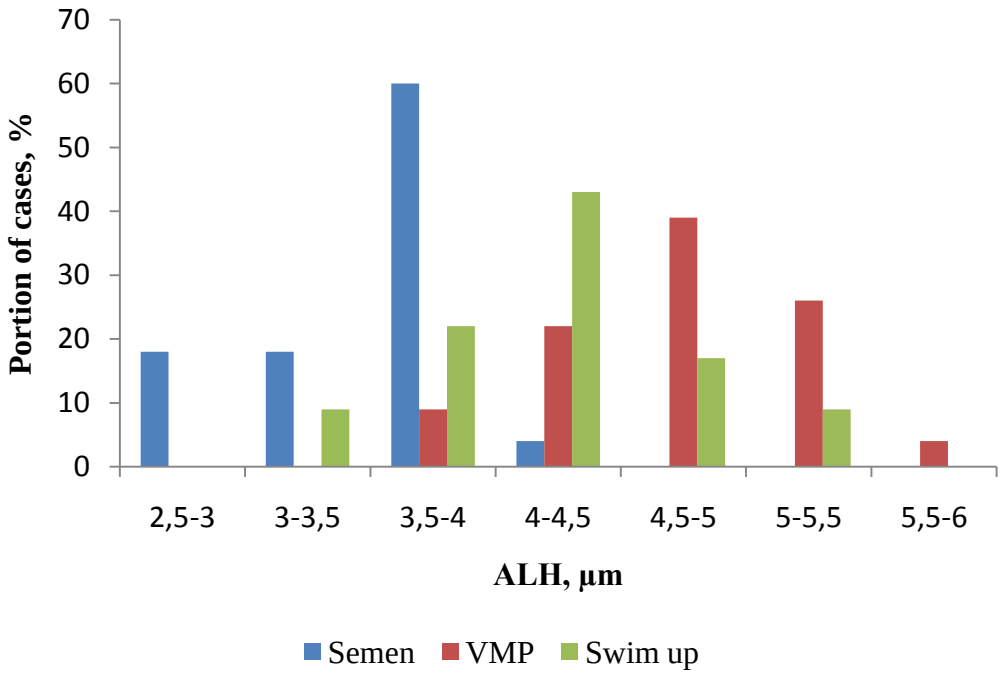


Figure 4.7 Distribution of ALH of spermatozoa in semen, spermatozoa recovered by the VMP method and swim up (n=23). The portion of cases when spermatozoa had higher ALH after preparation is significantly higher ($P<0.05$) than spermatozoa in semen. ALH of spermatozoa prepared by the VMP method was statistically significantly higher ($P<0.05$) than ALH of spermatozoa after swim up.

Average ALH after preparation by the VMP method was 4.7 μm and average ALH after preparation by swim up was 4.2 μm. There was a statistically significant difference ($P<0.05$) between these two methods of preparation.

Figure 4.8 shows the relationship between percentage of spermatozoa passed through viscous medium and ALH in semen. Correlation between ALH of spermatozoa in semen and percent of penetrated spermatozoa in these series of experiments was 0.76. Meanwhile, the correlation between ALH of spermatozoa in semen and percent of spermatozoa that migrated into overlaying medium was 0.39 and coefficient of determination was too low ($R^2= 0.18$) to build an accurate approximation.

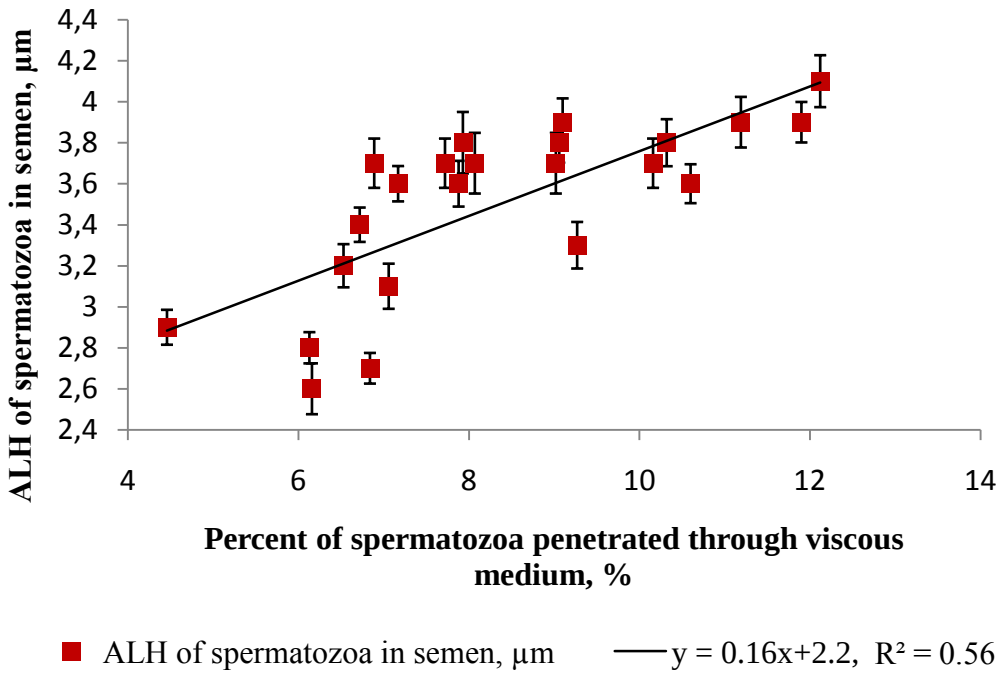


Figure 4.8 Relationship between the percentage of spermatozoa that penetrated through viscous medium from the drop of semen and ALH of spermatozoa in semen (n=23). Each vertical line indicates ± standard deviation of individual readings about the average value.

4.5 Discussion

In this chapter a novel method for sperm preparation was tested. The VMP technique is the first attempt to recover sperm that passed viscous medium. The main ideas that formed the basis of this method were: 1) physiological selection technique may improve fertilizing ability of spermatozoa (Perry *et al*., 1996), 2) stable and easy prepared viscous medium based on methylcellulose can be used as an alternative of cervical mucus. Concentration and motility characteristics of spermatozoa recovered by this technique were the first parameters that were compared with the characteristics of spermatozoa in semen and spermatozoa recovered by commonly used method – swim up. Compared to the raw semen, a significant improvement of sperm motility parameters was noted after sperm preparation by both methods, while the concentration of sperm in the prepared fraction was reduced. These characteristics were measured by CASA, which allows an objective and precise analysis.

4.5.1 Concentration

Analysis of the sperm concentrations in the fractions revealed a statistically significant difference between the VMP method and swim up. The concentration of spermatozoa prepared by the VMP method was significantly lower than the concentration of spermatozoa prepared by swim up. Despite good motility parameters, on average only 9% of spermatozoa were able to penetrate the viscous barrier. Although the yield was lower than in swim up, a sufficient number of motile spermatozoa were recovered suitable for IVF (~100 000 spermatozoa per oocyte are necessary) and ICSI. Although only normozoospermic semen samples

were included in these experiments, concentration and motility of sperm varied widely, allowing the model to be tested for different types of semen samples, and to build an accurate approximation. Recovery rate, i.e. the percent of sperm in semen that will be able to penetrate though viscous medium, was found to have a linear correlation to initial concentration in semen (r=0.57). Using that approximation it is possible to calculate the concentration of spermatozoa that will be able to penetrate viscous medium and estimate if the yield will be enough for IVF procedure.

The efficiency of that model for the samples of patients with oligozoospermia when the concentration of sperm is less than 15 M/ml is studied in Chapter 6.

4.5.2 Motility

Sperm motility in semen plays a significant role in the number of recovered spermatozoa after preparation with any technique (Henkel *et al.*, 2003). The correlation coefficients that influence on penetration ability are summarized in the table 4.1.

Table 4.1 Correlation coefficients between motility parameters and penetration ability of sperm in the VMP method.

Parameter	**Correlation coefficient**
Total motility	0.80
Progressive motility	0.50
VAP	0.72
VSL	0.64
VCL	0.72
ALH	0.76

A strong influence of sperm concentration in semen on the outcome of the VMP method is to be expected. Obviously the number of spermatozoa that can penetrate the viscous line is correlated positively with the number of spermatozoa in the semen at the beginning of viscous line. However, motility, velocity and ALH of spermatozoa in semen are also important in determining sperm penetration into viscous medium. The VMP method and swim up demonstrated good results in separating highly motile sperm from semen. In most of cases, there was no statistically significant difference between the methods of preparation. In motility characteristics ALH was the only parameter where a statistically significant difference was detected between two methods of preparation, suggesting that penetration through viscous medium requires vigorous movement of spermatozoa with high magnitude of sperm head oscillations about its track to pass through it.

Thus, the VMP method is a very gentle technique, especially if compared with methods that require centrifugation. This method can be used in order to recover a clean fraction of highly motile spermatozoa. Although currently, in this configuration, this technique is restricted to ejaculates of high sperm count and good motility, and the yield is lower than in swim up, a sufficient number of motile spermatozoa can be recovered for IVF procedure. The question if this method isolates sperm with better morphology and functional characteristics will be answered in the next chapter.

Chapter 5 Morphology and Functional Characteristics of Spermatozoa Recovered by the Viscous Medium Penetration Method

5.1 Introduction

As mentioned above, traditional parameters of semen analysis, such as concentration, motility and viability of spermatozoa have little predictive value for sperm fertilizing capacity *in vitro* (Liu & Baker, 1992). In order to find out the characteristics that may influence on fertilization ability of sperm further studies were carried out. Previously it was shown, that progesterone-induced increase of sperm Ca^{2+} is significantly correlated with egg-penetrating ability of spermatozoa from fertile but not infertile men (Alasmari *et al*., 2013; Shimizu *et al*., 1993) and significantly correlated with the acrosome reaction and fertilization rate (Krausz *et al*., 1995, Fig.5.1).

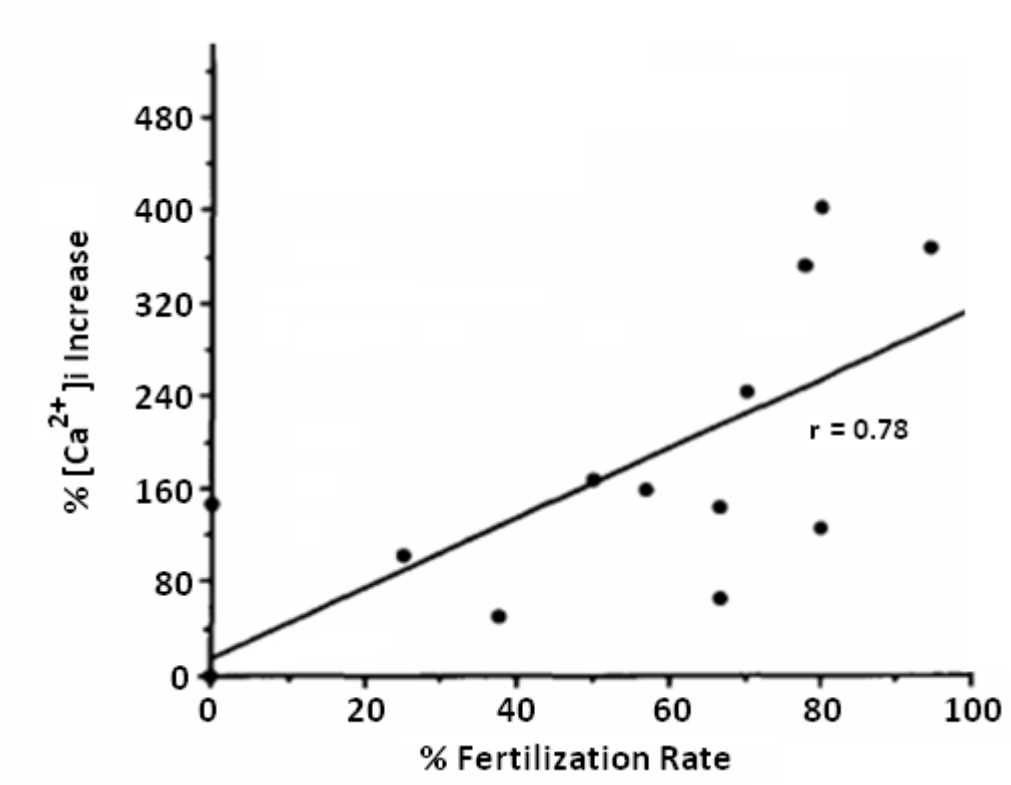


Figure 5.1 Correlation curve between the fertilization rate and the increase of sperm Ca^{2+} following challenge with 0.1 µg/ml progesterone (from Krausz *et al*., 1995).

Progesterone which is secreted by the cumulus cells of the oocyte, is able to enhance important sperm functions such as acrosome reaction, hyperactivation and sperm-oocyte penetration by activating cationic channels of sperm (CatSper) and elevating sperm intracellular Ca^{2+} (Lishko *et al.*, 2011). Thus, Ca^{2+} response to progesterone provides essential information about sperm fertilization ability for *in vivo* fertilization, intrauterine insemination and IVF. However, this method does not indicate the DNA integrity or chromosomal abnormalities of each individual spermatozoon. This information is essential in ICSI, when only one spermatozoon must be injected into the oocyte.

The fact, that ICSI using non capacitated and acrosome-intact spermatozoa, is successful proves that the genetic information carried by these cells is fully competent to support complete development once it has reached the oocyte's cytoplasm (Fraser, 1998). However, during capacitation and the acrosome reaction *in vivo*, only a small proportion of the sperm in the ejaculate will reach the site of fertilization in a physiologically appropriate state to be able to fertilize an oocyte (Bedford *et al.*, 1970; Harper *et al.*, 1982). Thus, *in vivo* there are a number of selective pressures placed on the initial population of sperm and these may be important in helping to select the 'fittest' spermatozoa for fertilization. No such selection is imposed on the spermatozoa used for ICSI. Although with ICSI it is possible to bypass all natural barriers such as acidic environment in the vagina, cervical mucus and the long distance to the egg, but fertilization with DNA-fragmented sperm is possible. In the case of this event the fertilization rate, embryo quality and pregnancy rate will be reduced. It was shown that in teratozoospermic semen samples even morphologically normal sperm may

contain highly fragmented DNA (Avendaño *et al.*, 2009). Recently, a method of sperm selection that can potentially reduce the chances of fertilization with high fragmented DNA sperm in IVF was developed. This method is based on the research that hyaluronic acid (HA) binds to mature sperm with intact acrosome (Huszar *et al*., 2003). Gabor Huszar studied various biochemical markers of human sperm maturity and function. He measured sperm creatine kinase (CK) – a key enzyme in the synthesis and transport of energy – and found that men with low sperm concentrations, who have increased incidences of infertility, show high levels of sperm CK activity. When he studied each individual sperm, it was found that high sperm CK activity is a related with high CK and cytoplasmic protein concentrations. These findings suggested that this is a defect of sperm development in the last phase of spermiogenesis, when excess cytoplasm is normally extruded. Thus, sperm with high CK content, an indication of surplus cytoplasm, have not completed cellular maturation. Later, it was shown a close correlation between the decline of sperm CK activity and increase in testis-expressed protein HspA2. This protein is synthesized in two waves of expression. The first wave occurs during meiosis, as HspA2 is part of the synaptonemal complex. The second wave of major expression occurs simultaneously with cytoplasmic extrusion, in terminal spermiogenesis. Finally, it was found that immature sperm with retained cytoplasm, high CK content, and low expression of HspA2 are apparently deficient in the zona-binding site. Similar to the binding observed between sperm and the zona pellucida, developmentally mature sperm bind to crosslinked hyaluronan gels and to hyaluronan chemically attached to a base structure.

Many clinical studies with approximately 2500 patients in total showed that HA binding sperm selection improves fertilization rate, embryo quality and implantation (Parmegiani *et al*., 2010; Tarozzi *et al*., 2009; Worrilow *et al*., 2013; Ye *et al*., 2006). However, some authors report that there was no improvement in embryo quality by using HA bound spermatozoa (Kovacs *et al*., 2011). Majumdar *et al*. (2013) report that although there were no differences in fertilization rate between visual and HA selections of sperm, a lower pregnancy loss was observed in the group where HA binding sperm selection was used.

Another important benefit of using HA is the reduction of chromosomal abnormalities in comparison with non-selected sperm. Combined studies of sperm shape and chromosome probes demonstrated that only sperm morphology does not aid in selection of haploid spermatozoa (Celik-Ozenci *et al*., 2004; Ryu *et al*., 2001; Sun *et al*., 2006). Aneuploidy of spermatozoa is correlated with implantation failure and miscarriages although fertilization rate can be rather high (~70%) (Carrell *et al*., 2003; Pfeffer *et al*., 1999).

Currently, there are no methods for selection of spermatozoa without chromosomal abnormalities. However, the percentage of spermatozoa that bound with HA is correlated with the number of chromosome abnormalities and therefore using HA-selected spermatozoa, it is possible to reduce the frequency the chromosomal disomy and diploidy 4- to 6-fold compared with semen sperm fractions (Huszar *et al*., 2007; Mokánszki *et al*., 2012).

Thus, the percentage of spermatozoa that were bound with HA is a good marker for fertilization ability of these spermatozoa. In this case it is interesting to study

the portion of spermatozoa that will be able to bind with HA after preparation by the VMP method.

5.2 Aims and experimental design

The main aim of this chapter is to compare morphology, Ca^{2+} response to progesterone and the ability of sperm to bind with HA of sperm recovered by the VMP method and by swim up.

Firstly, the correlation between sperm morphology and sperm penetration ability in the VMP system that was determined as the ratio

$$100 \times \frac{concentration\ of\ recovered\ spermatozoa}{concentration\ of\ spermatozoa\ in\ semen}\%$$

was studied. Second, the percentages of normal sperm and percentages of defects after swim up and the VMP were compared. Third, the increases of Ca^{2+} of sperm recovered by swim up and the VMP method were studied and compared. Fourth, the percentages of spermatozoa that were able to bind with HA after the VMP and swim up were studied and compared.

5.3 Materials and methods

20 semen samples were examined from 17 different donors. The portion of spermatozoa with normal morphology after preparation by the VMP method was compared with spermatozoa after swim up and spermatozoa in raw semen. The results were expressed as the percentage of normal sperm observed on each slide. To be classified as normal, a sperm must have a smooth, oval head with a well-defined acrosome incorporating 40% – 70% of the sperm head, no neck, midpiece or tail defects, and no cytoplasmic droplets that are more than one-half the size of

the sperm head (WHO, 2010). According to the WHO, sperm with borderline morphologies were counted as abnormal. 200 fixed and stained cells (see 2.2.3) in each case in each group were studied: percentage of normal forms, percentages of defects in head, midpiece, tail, percentage of spermatozoa with cytoplasmic droplets. After that teratozoospermia index (TZI) for each case in each group was calculated. TZI is defined as the number of abnormalities per abnormal spermatozoon. All abnormal spermatozoa had from one to four abnormalities, including head, midpiece, tail defects and the presence of cytoplasmic droplets. The classification of spermatozoa for TZI was carried out on a laboratory counter. The spermatozoa are recorded as normal or abnormal and distributed into specific groups (head, midpiece and tail defects or cytoplasmic droplets groups). The total number of abnormalities is then added together and divided by the number of abnormal sperm.

After an initial assessment of volume, the number of motile sperm/ml, motility and hyaluronic binding score, semen sample was split into two fractions and prepared by swim up and the VMP method. The processed sperm suspensions were scored for the number of motile sperm/ml, percentage of motility and hyaluronic binding score. After evaluation of these characteristics, the fractions were prepared for the measurements of Ca^{2+} response for progesterone as described in 2.2.4. Due to technical limitations it was not possible to evaluate intracellular Ca^{2+} for every individual sample. In order to detect a signal, only cases where more than ~10 M cells recovered by the VMP method were tested.

5.4 Results

5.4.1 Morphology

The Figure 5.2 presents the percentages of morphologically normal spermatozoa in semen and spermatozoa recovered by the VMP method and swim up in 20 experimental cases.

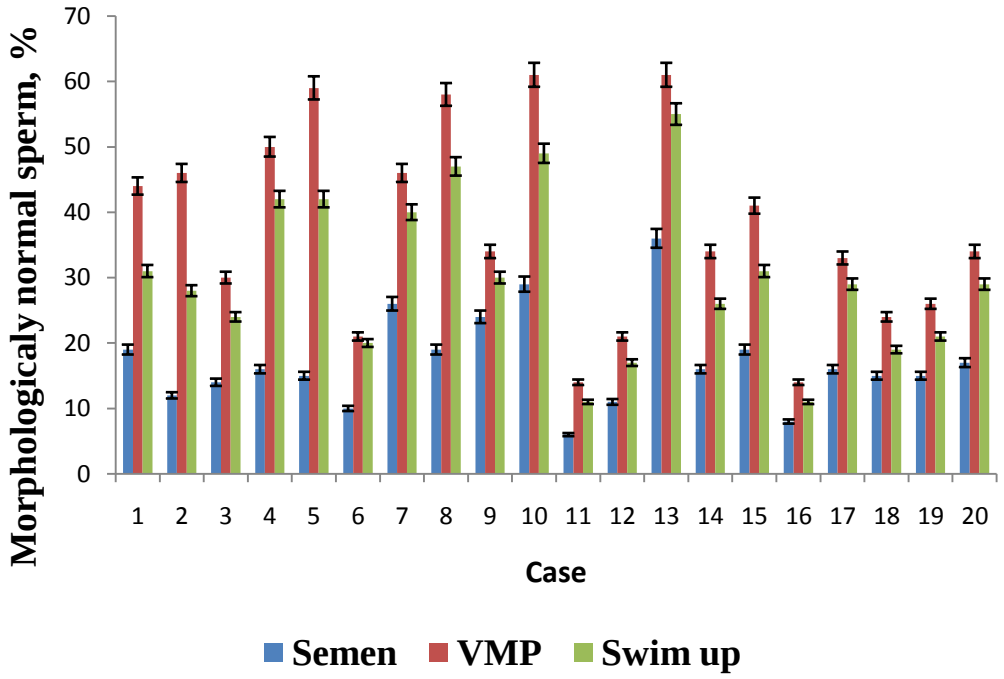


Figure 5.2 Morphology of spermatozoa in semen, and spermatozoa recovered by the VMP method and swim up (number of semen samples: n=20). Each vertical line indicates ± standard deviation of individual readings about the mean. The percent of morphologically normal spermatozoa was significantly higher ($P<0.05$) after preparation than percent of morphologically normal spermatozoa in semen. The difference between the methods of preparation was not statistically significant ($P=0.09$).

The percentage of morphologically normal spermatozoa in semen in all cases met the WHO criteria for normozoospermia (Table 1.1) and varied from 6% to 36% with an average value of 17%. Prepared spermatozoa had significantly ($P<0.05$) higher percentage of morphologically normal spermatozoa: in 80% of all cases the percentage of morphologically normal sperm after preparation by all methods was higher than 17%. The average values of morphologically normal spermatozoa after preparation by swim up and the VMP method were 30% and 38% respectively. However, no statistically significant difference in percentage of morphologically normal spermatozoa was found between the methods of preparation ($P = 0.09$).

The Figure 5.3 presents the TZI of spermatozoa in semen and spermatozoa recovered by the VMP method and swim up in 20 experimental cases. The TZI of sperm in semen varied from 1.15 to 1.64 with an average value of 1.4. Prepared spermatozoa had significantly ($P<0.05$) lower TZI and there was a statistically significant difference between the methods of preparation ($P<0.05$). The average TZI of sperm recovered by the VMP method and by swim up were 1.13 and 1.21 respectively.

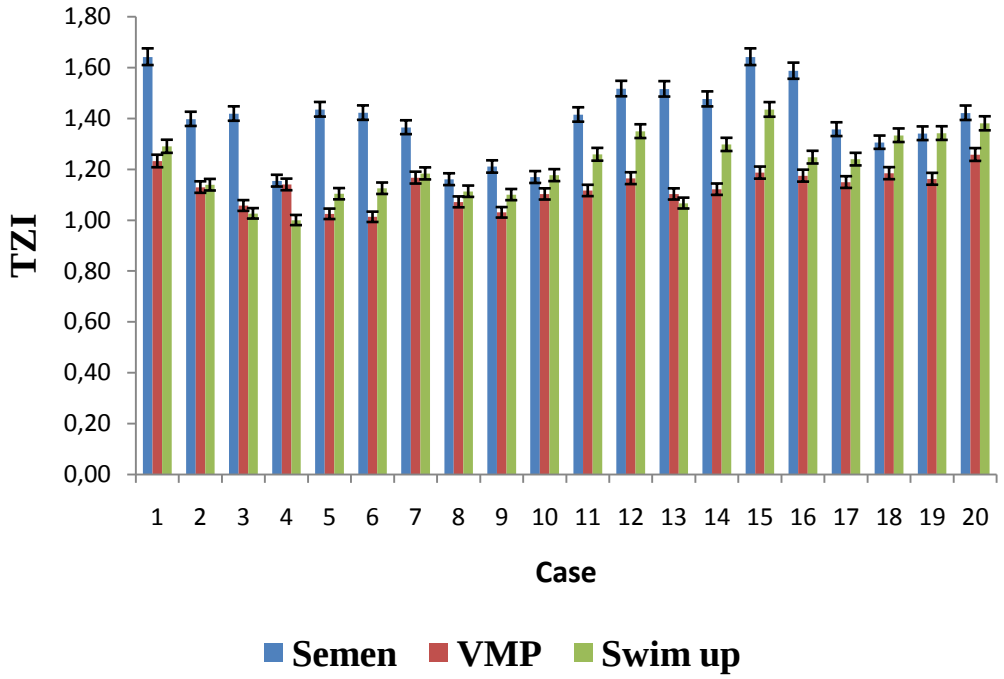


Figure 5.3 Teratozoospermia index (TZI) of spermatozoa in semen, and spermatozoa recovered by the VMP method and swim up (n=20). Each vertical line indicates ± standard deviation of individual readings about the mean. TZI of spermatozoa was significantly lower (P<0.05) after preparation than TZI of spermatozoa in semen. The difference between the methods of preparation was statistically significant (P<0.05).

Figure 5.4 shows the average percentages of normal spermatozoa and the percentages of different defects in sperm in semen, after the VMP method and swim up. The spermatozoa with normal morphology and spermatozoa with defects in head, midpiece, tail and with cytoplasmic droplets were compared in the semen, after the preparation by the VMP method and swim up. It was found that percentage of sperm with defects was significantly lower (P<0.05) after preparation of semen. The statistical significant difference between the VMP method and swim up was observed in the groups of spermatozoa with the defects in midpiece (P<0.05) and with the cytoplasmic droplets (P<0.05). There was no

statistically significant difference in the groups of spermatozoa with the defects in head (P=0.28) and tail (P=0.11).

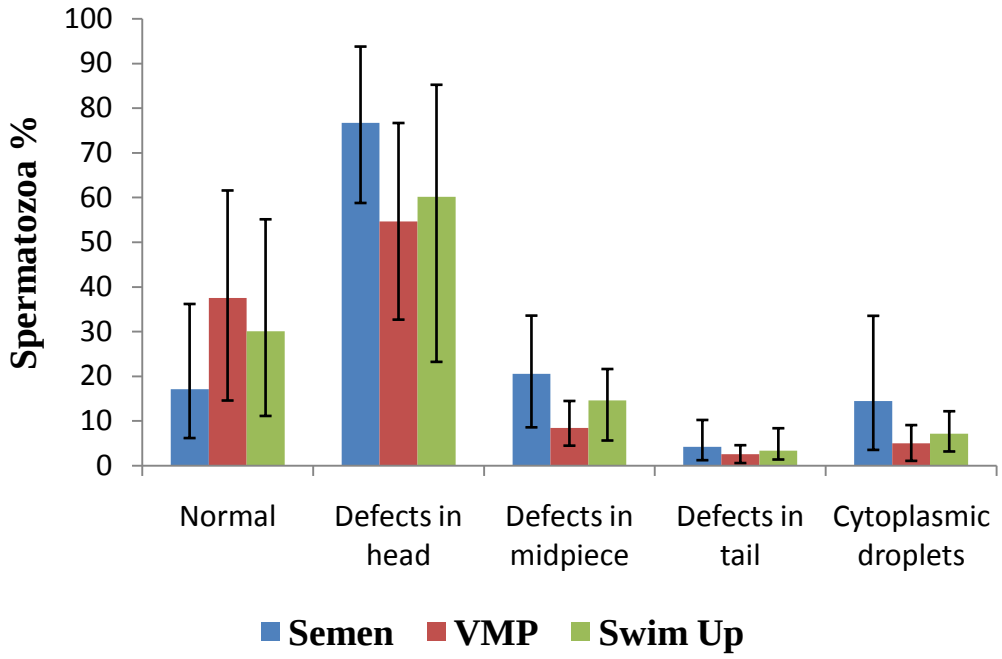


Figure 5.4 Average percentages of normal spermatozoa and the percentages of different defects of sperm in semen, and sperm recovered by the VMP and swim up (20 cases). Each vertical line indicates ± standard deviation about the mean. Statistical difference (P<0.05) was observed between the spermatozoa in semen and after preparation. No statistically significant difference was observed between the methods of preparation in the groups of normal sperm (P=0.09), with defects in head (P=0.28), with defects in tail (P=0.11). The number of sperm with defects in midpiece and with cytoplasmic droplets was statistically lower after the VMP method than after swim up (P<0.05).

In order to compare the relative changes in sperm morphology after the preparation of semen by the VMP method and swim up, the absolute values of the number of normal forms, sperm with defects in head, midpiece, tail and the number sperma with cytoplasmic droplets were divided by the absolute values of the same sperm characteristics in semen. For example, the average percent of normal spermatozoa in semen was 17%. After preparation by the VMP method, the average was 38%. The ratio of 38% to 17% indicates the change in portion of morphologically normal spermatozoa after preparing semen by the VMP method. The results are presented in figure 5.5.

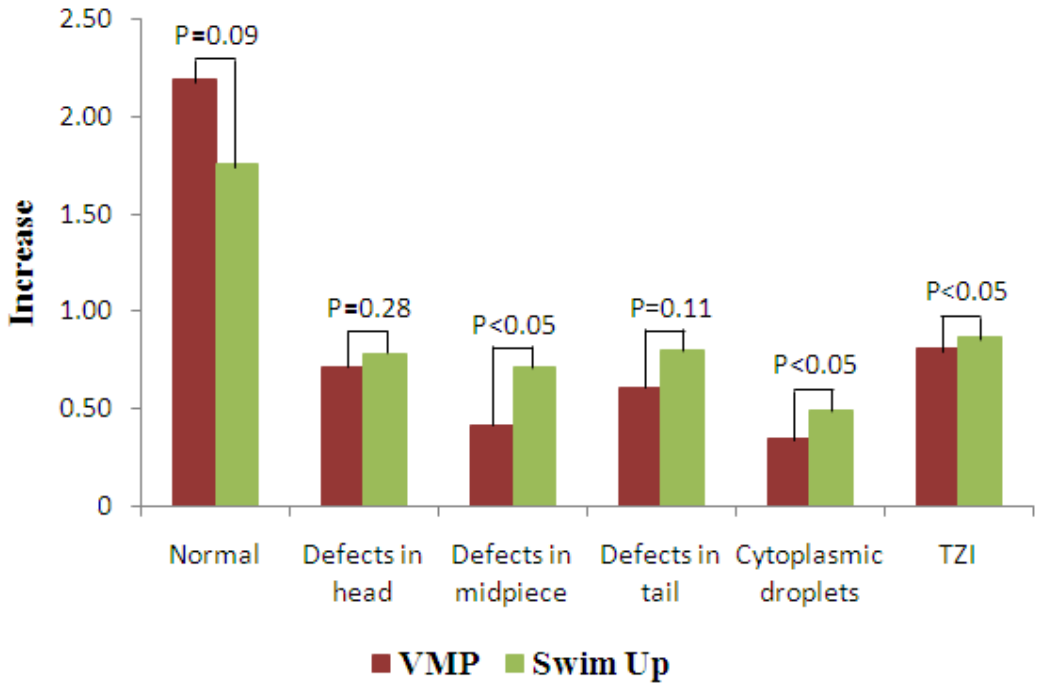


Figure 5.5 Changes in the number of normal spermatozoa, sperm with different defects and TZI after preparation of semen by the VMP method and swim up.

Figure 5.6 shows the relationship between the penetration rate and percent of morphologically normal spermatozoa in semen. Correlation between morphologically normal spermatozoa in semen and penetration rate in these series of experiments was 0.82.

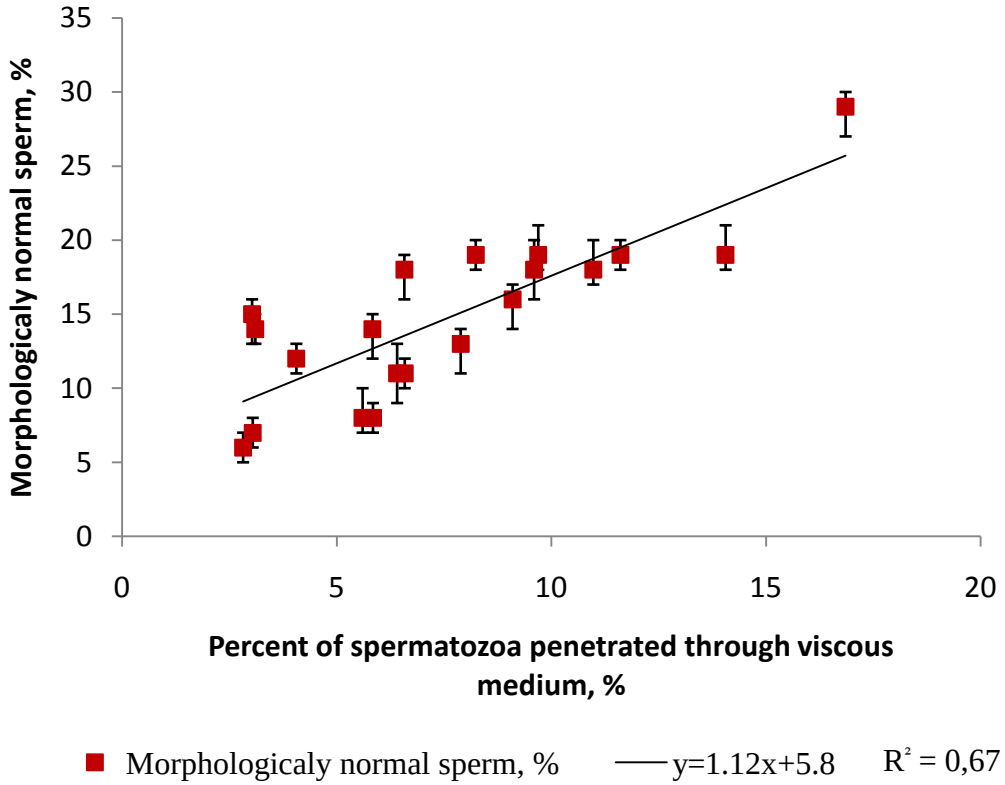


Figure 5.6 Relationship between the percentage of morphologically normal spermatozoa in semen and penetration (n=20). Each vertical line indicates ± standard deviation of individual readings about the mean.

5.4.2 Calcium response to progesterone

Calcium response to progesterone is presented in the Figure 5.7. The concentration of spermatozoa in all studied cases for each group was adjusted to the same level (~10 M/ml). An average increase of signal after stimulation of

spermatozoa with progesterone was 2.4 and 2.6 for spermatozoa recovered by the VMP method and swim up respectively.

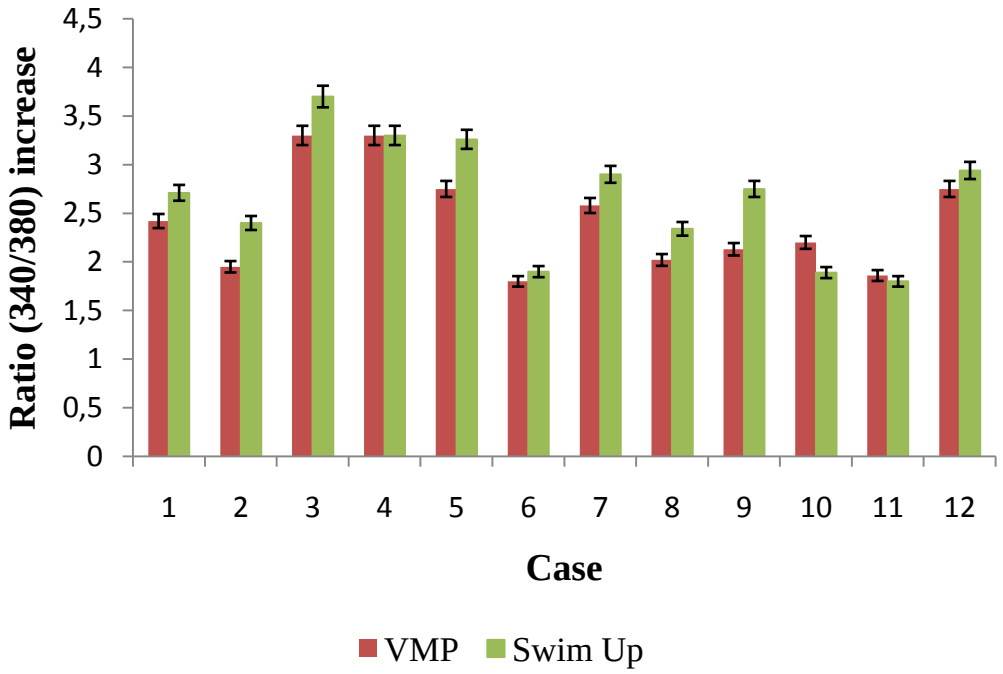


Figure 5.7 The ratio of fluorescence increase at 340 nm and 380 nm showing calcium response to progesterone of spermatozoa by the VMP method and swim up (n=12). Each vertical line indicates ± standard error of individual readings about the mean. The difference between the methods of preparation was not statistically significant (P>0.05).

5.4.3 Hyaluronic acid binding

The comparison of spermatozoa that were able to bind with the hyaluronic acid treated slide in semen and after preparation by swim up and the VMP method is presented in the Figure 5.8. The percentage of spermatozoa bound with HA was significantly higher after preparation of semen (P<0.05). There was also a statistically significant difference between the methods of sperm preparation

($P<0.05$). Therefore spermatozoa that were recovered by the VMP method have a greater capacity to bind to HA than spermatozoa recovered by swim up.

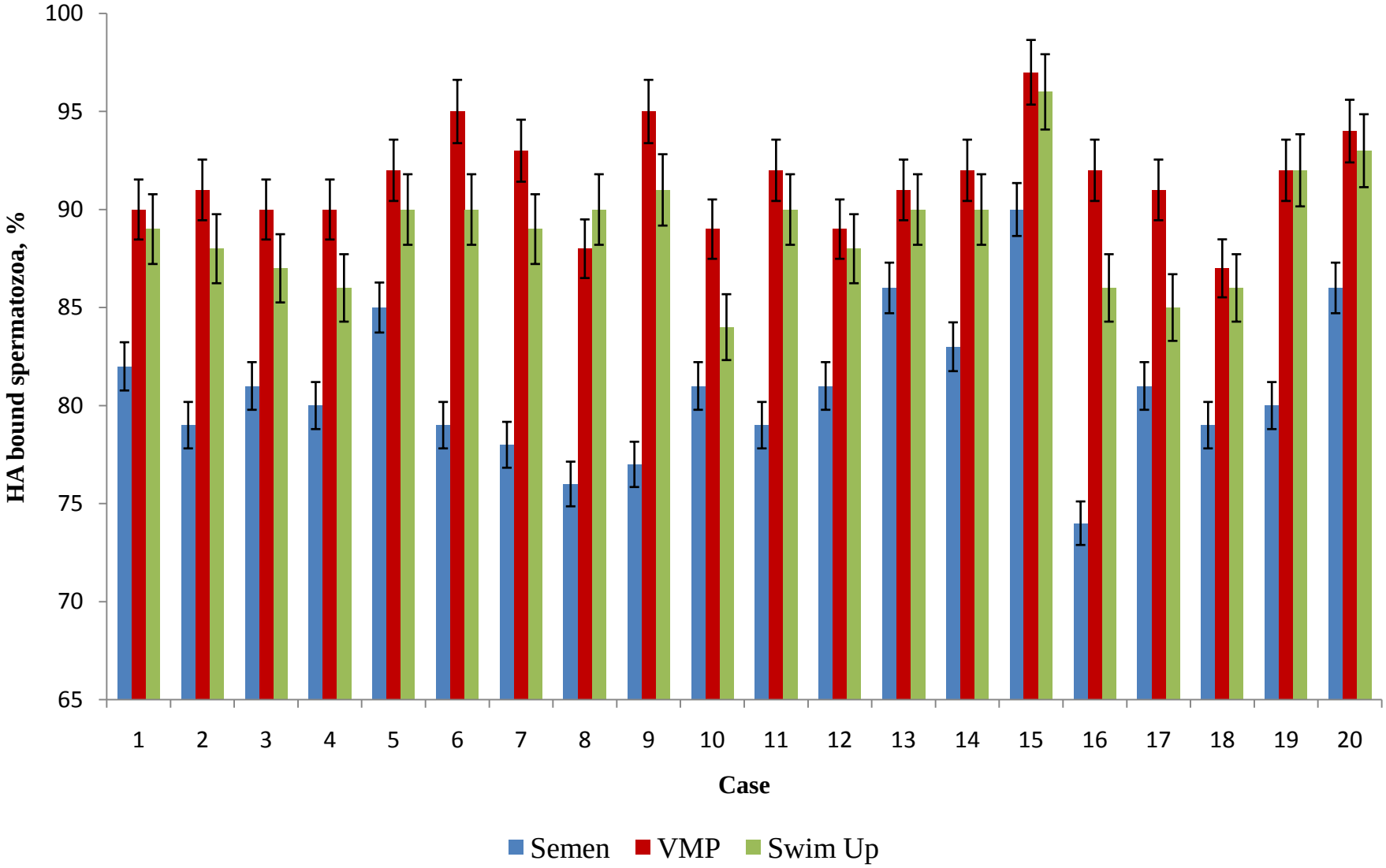


Figure 5.8 Percentage of hyaluronic bound spermatozoa in semen and after preparation by VMP and swim up (n=20). Each vertical line indicates ± standard deviation of individual readings about the mean. The difference between the methods of preparation is statistically significant (P<0.05).

Figure 5.9 shows the relationship between the penetration rate and percent of HA bound sperm in semen. Correlation between these characteristics was 0.76.

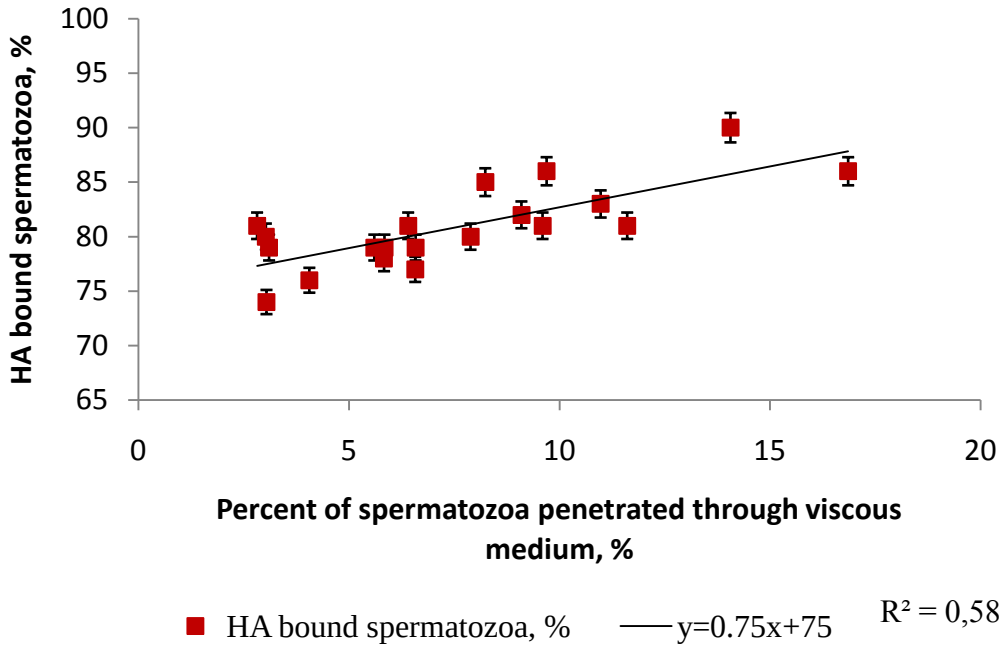


Figure 5.9 Relationship between the percentage of spermatozoa bound with hyaluronic acid in semen and sperm penetration. Each vertical line indicates ± standard deviation of individual readings about the mean.

5.5 Discussion

5.5.1 Morphology

It was found that the VMP method characterized by a good ability to select morphologically normal spermatozoa. The results showed a high correlation (r=0.82) between the penetration rate of spermatozoa in the VMP method and morphologically normal sperm in semen. Therefore, it is possible to predict the

number of sperm that will be capable to penetrate the viscous medium if the information about their morphology is available.

Although statistically significant difference in the number of morphologically normal sperm between two methods of preparations was not determined (P=0.09), it was found that there was a statistical significant difference (P<0.05) between TZI of spermatozoa recovered by VMP method and TZI of spermatozoa recovered by swim up. Average TZI of sperm that were able to penetrate viscous medium was 1.13, after swim up 1.21. That means that spermatozoa recovered by the VMP method have fewer defects in general than spermatozoa prepared by swim up. The most frequent abnormalities of sperm in semen were observed in head and midpiece. However, after preparation by the VMP method a significant reduction (P<0.05) of spermatozoa with defects in midpiece was observed. It is possible to assume, that viscous medium is a very efficient barrier for spermatozoa with defects in the midpiece which is the site of mitochondria in sperm. Such defects do not allow sperm to penetrate through dense structure of viscous medium. The same effect was observed by Mortimer *et al*., (1982) when they studied the morphological status of spermatozoa penetrated into the cervical mucus.

5.5.2 Calcium response to progesterone

Krausz *et al.* (1995) discovered that the responsiveness to progesterone of spermatozoa, measured as increase of Ca^{2+} can be a good predictive marker for fertilization rate and IVF outcome. The measurement can be easily and quickly

performed in most laboratories; it utilizes widely available reagents and requires only a spectrofluorimeter.

The results of the experiments showed that spermatozoa that were able to get through viscous medium have the same sensitivity to progesterone as spermatozoa recovered by swim up. In all cases, when it was possible to recover enough cells to carry out the experiment, a significant increase of the signal after stimulation was observed. However, the difference in ratio (340/380) between these two groups of spermatozoa was not statistically significant ($P>0.05$). The kinetics of the response were similar in the sperm which were recovered by the VMP method and swim up sperm. It is possible to assume that CatSper is not adversely affected during sperm interaction with the methylcellulose solution. It is also interesting that there was no significant correlation between any of the motility parameters and Ca^{2+} increase in the fractions of sperm prepared by the VMP technique or swim up.

5.5.3 Hyaluronic acid binding

The percentage of spermatozoa bound with HA was significantly higher after preparation of semen ($P<0.05$). Also, there was a statistically significant difference between the methods of sperm preparation ($P<0.05$). Spermatozoa that had passed through viscous solution had a statistically greater tendency to bind to HA than spermatozoa recovered by swim up. Therefore, it is possible to assume that spermatozoa recovered by the VMP method are more mature, have more compacted chromatin and firm membrane than spermatozoa recovered by swim up. Most of immature sperm and sperm with labile membrane and lipid structure

cannot pass through the dense structure of viscous medium. This assumption can be confirmed by the relationship between the penetration rate and the percentage of bound sperm in semen (Fig. 5.9). This linear dependence shows that the higher percentage of bound spermatozoa is observed in semen, the higher portion of them will be able to pass through viscous medium and be recovered from the drop of STF.

In summary, the VMP method could potentially enhance the selection of spermatozoa with intact DNA and less chromosomal abnormalities for IVF/ICSI.

Chapter 6 Semen Preparation for IVF in Severe Male Factor Infertility by the Viscous Medium Penetration Method

6.1 Introduction

Currently, IVF/ICSI is the only treatment for cases of infertility due to male factor. In 58.5% of semen samples examined in Assisted Conception Unit, Ninewells hospital in 2012 sperm concentration, motility or morphology were below the WHO criteria for normozoospermia (Fig. 6.1, data were processed by L. King). 51% of semen samples obtained from the patients had at least one sperm characteristic that was abnormal. Despite intensive research, there are almost no available drugs for improvement of sperm characteristics in semen (Alasmari *et al.*, 2013; Kamischke & Nieschlag, 1999; Safarinejad & Safarinejad, 2009). Therefore, currently patients with sperm dysfunction have to rely on Assisted Reproductive Techniques (ART).

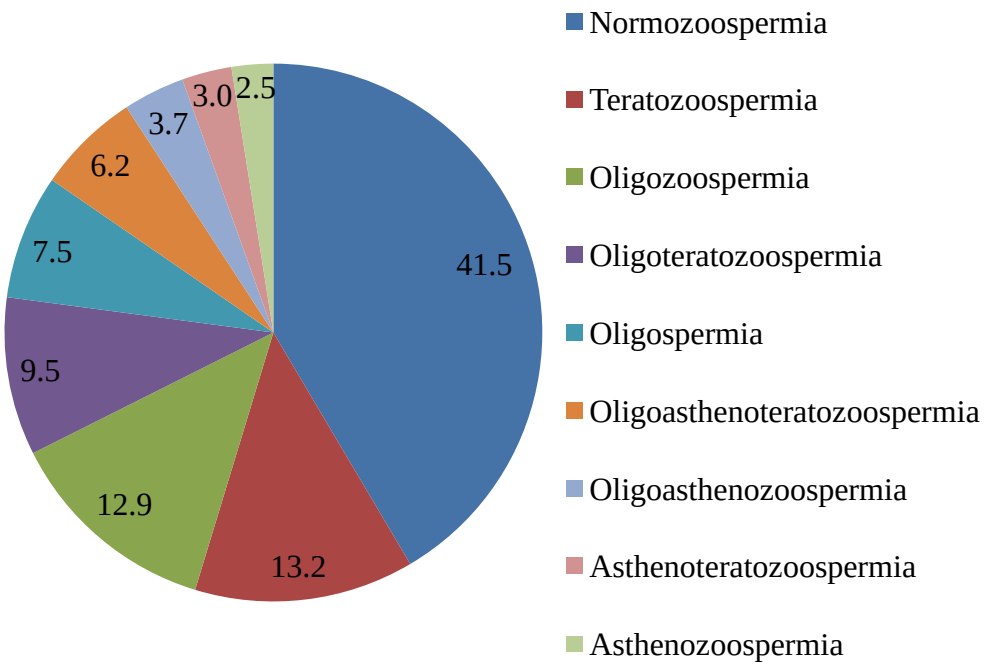


Figure 6.1 Distribution of 552 semen samples in Assisted Conception Unit, Ninewells hospital, 2012.

Semen preparation and sperm selection are especially important for the oligoasthenoteratozoospermia patients when sperm concentration, motility and morphology are reduced. Although there is a wide variety of semen preparation protocols, not all of them are capable to recover sperm from patients' samples. Application of these methods for poor semen samples cannot guarantee that enough sperm will be recovered. Reduced concentration of spermatozoa in a prepared fraction is correlated with low fertilization rate in IVF (Gellert-Mortimer *et al*., 1988; Wolf *et al*., 1984). In these cases, such methods as swim up and sedimentation are not very effective in recovering clean fractions of motile spermatozoa (Al Hasani *et al.*, 1995). Simple washing does not remove cell debris and leukocytes from semen. Density gradient centrifugation is a very effective technique, but it can provide with high number of immotile sperm in the yield in poor samples (Evenson *et al.*, 2002).

In order to identify the efficiency of the viscous medium penetration (VMP) method to isolate cells from the poor samples, semen with low concentration, motility and poor morphology of sperm were used. The VMP method can facilitate the sperm selection and, therefore, can potentially reduce the time of ICSI procedures.

6.2 Aims and experimental design

The first aim is to report the results of testing the ability of the VMP method to recover a fraction of motile spermatozoa from patients with oligoasthenoteratozoospermia. The second aim is to compare the capacity to

recover sperm from normozoospermic donors' and oligoasthenoteratozoospermic patients' samples by the VMP method.

The study group comprised 20 males with low sperm quality. Semen obtained from men of this group was prepared by the VMP method. The characteristics of recovered spermatozoa were compared with the characteristics of sperm recovered by the VMP from donors which described in Chapters 4 and 5.

6.3 Materials and methods

In these experiments semen samples with the sperm concentration less than 15 M/ml, total percent of motile sperm lower than 40%, percent of progressive motile sperm lower than 32% and morphologically normal forms of spermatozoa less than 4% were used. Concentration, motility and morphology characteristics of sperm in the samples used in the experiments for this chapter are presented at the Figures 6.2, 6.3 and 6.4 respectively.

In all cases the concentration of recovered spermatozoa was less than $\leq$ 2M/ml. This concentration can affect CASA and FLUOstar readings (for measuring motility characteristics and intracellular Ca^{2+} levels, respectively), and consequently make attaining comparable and reliable data between samples very difficult. In order to evaluate the concentration and motility of recovered sperm more accurate FastRead 102 counting chambers were used (Gunetti *et al*., 2012). After evaluating the concentration, non-motile sperm were first counted within an area of 16 squares in the center of the grid. Then moving sperm were counted. The procedure was repeated in several areas and percentage of motile sperm was calculated.

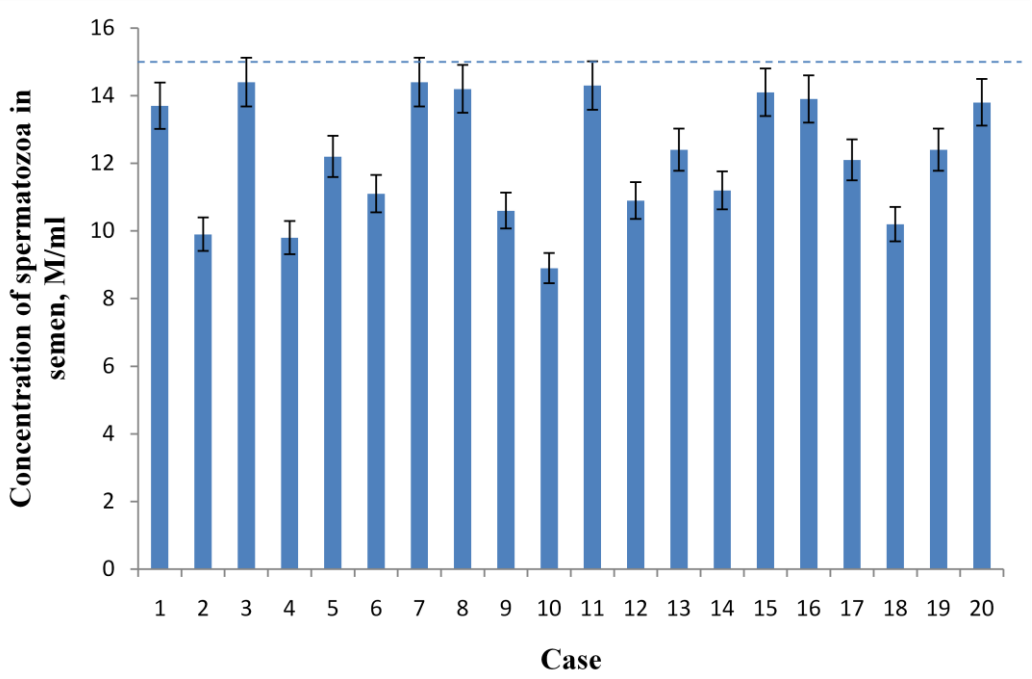


Figure 6.2 Concentrations of spermatozoa in semen samples in experimental cases. The result shown is the mean ± standard deviation for concentration of semen samples from 20 patients. Only semen samples with the concentration lower 15 M/ml were tested. The dashed line shows the minimum level of sperm concentration in normozoospermic sample according to the WHO criteria (WHO, 2010).

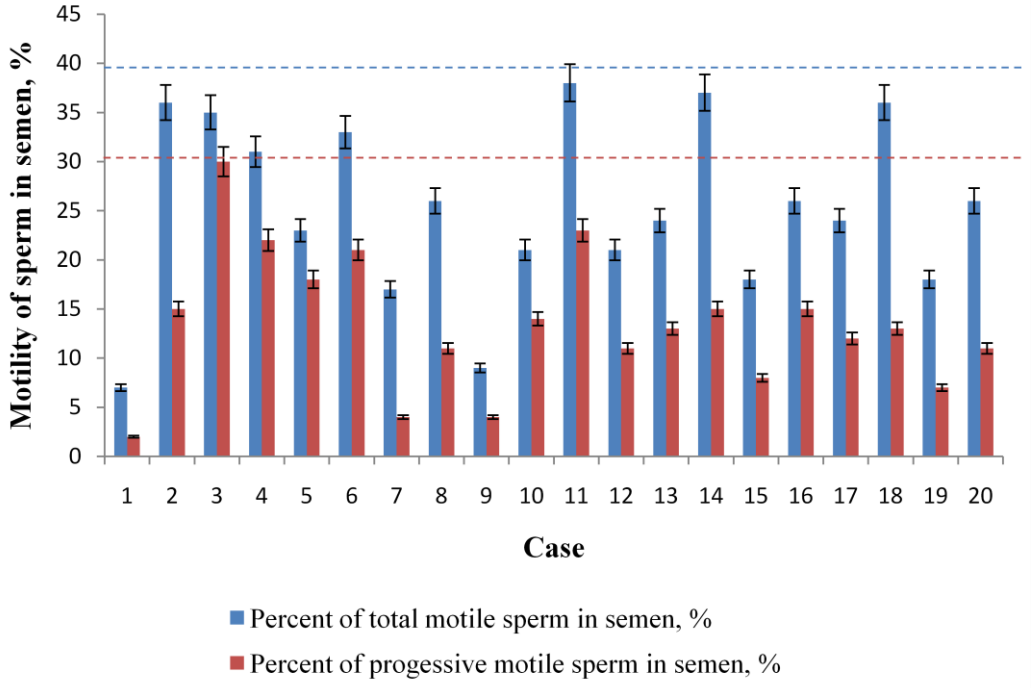


Figure 6.3 Percentages of total and progressive motile spermatozoa in experimental cases. The result shown is the mean ± standard deviation for motility

of spermatozoa in semen samples from 20 patients. The dashed lines show the minimum levels of total (blue) and progressive (red) percentage of motile sperm in normozoospermic sample according to the WHO criteria (WHO, 2010).

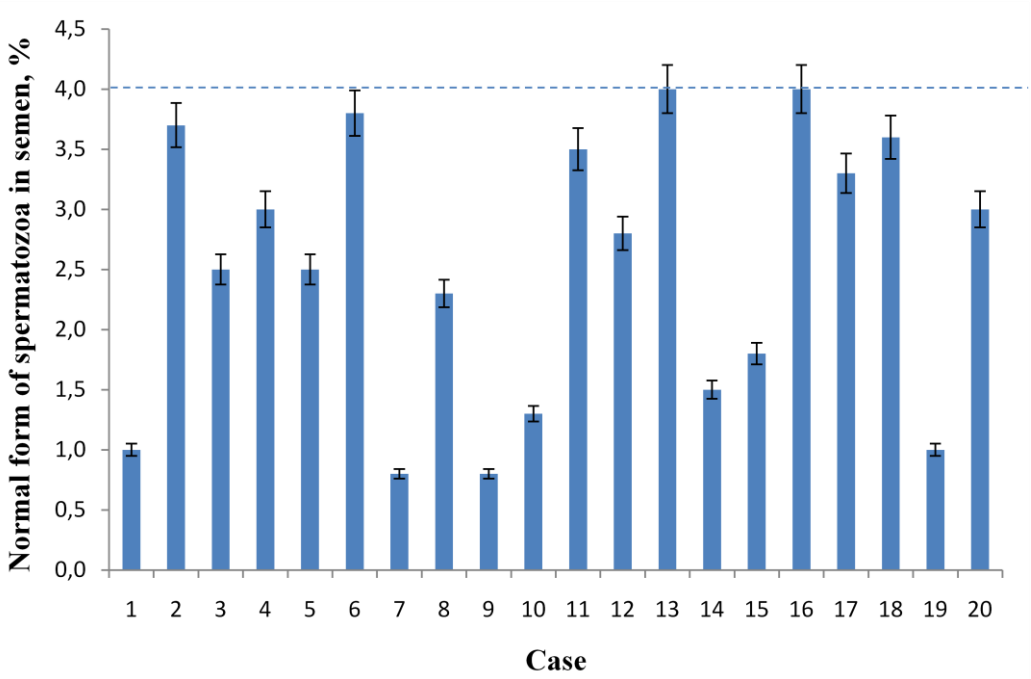


Figure 6.4 Percentage of morphologically normal forms of spermatozoa in semen samples in experimental cases. The result shown is the mean ± standard deviation for morphology of spermatozoa in semen samples from 20 patients. The dashed line shows the minimum level of sperm morphology in normozoospermic sample according to the WHO criteria (WHO, 2010).

6.4 Results

6.4.1 Concentration of recovered spermatozoa

Previously, the VMP method was tested on donors and it was shown that it is possible to recover a clean fraction of highly motile spermatozoa. This model was built for the samples with sperm concentration higher than 35 M/ml, and a good correlation between the concentration of sperm in semen and the concentration of sperm after VMP was found (r = 0.92, Figure 6.5). The relationship between the concentration of spermatozoa in semen and the concentration of recovered sperm in the case of oligoasthenoteratozoospermia is presented at the Figure 6.6.

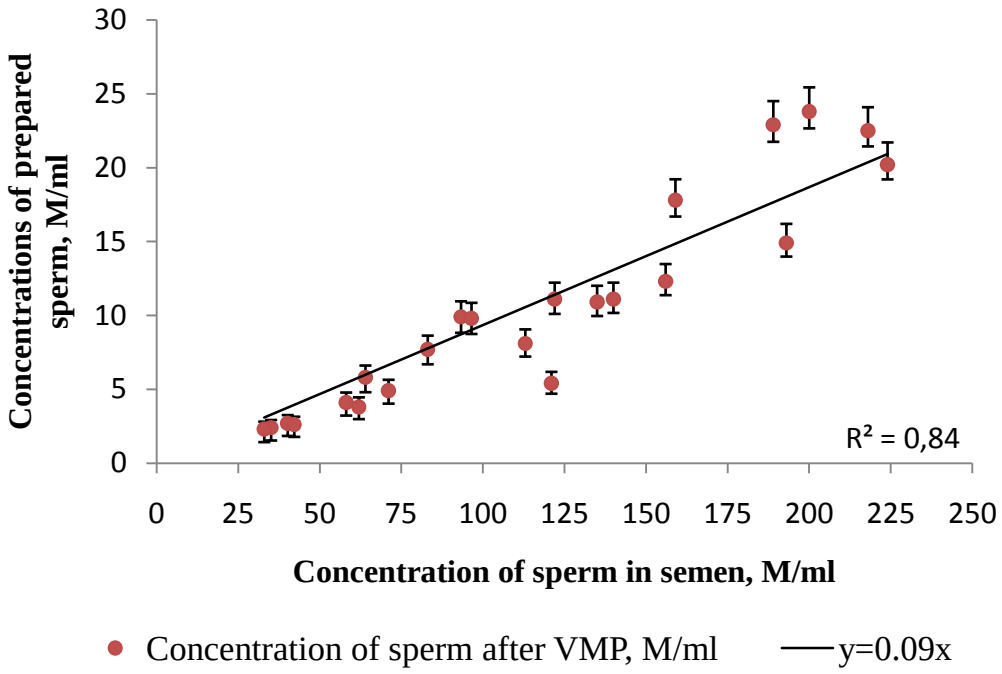


Figure 6.5 Concentration of spermatozoa recovered from donors' semen samples by the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 23 semen samples.

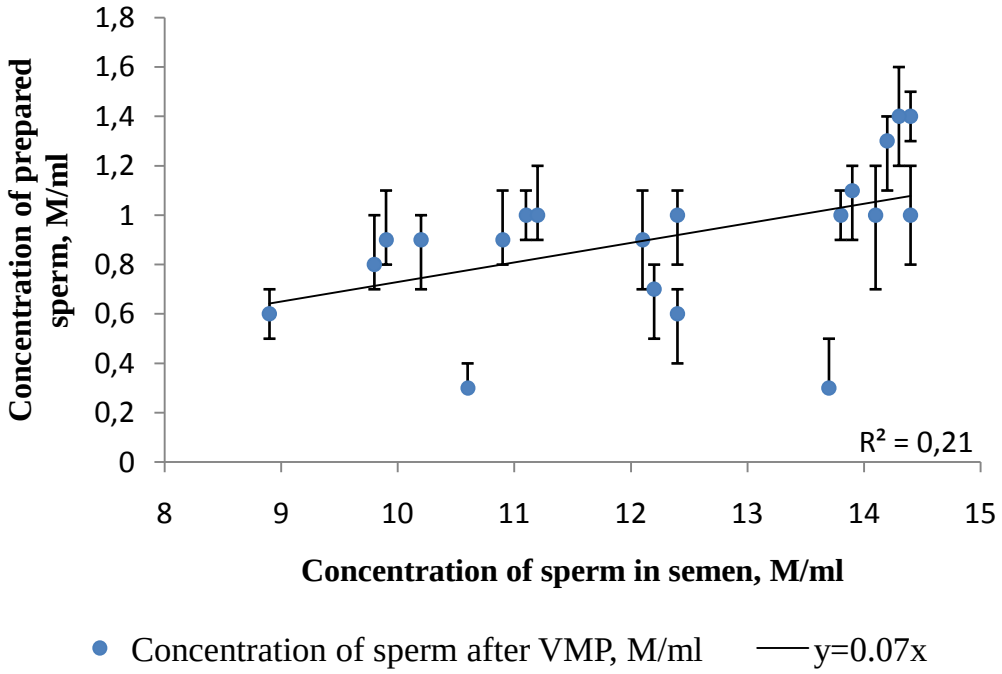


Figure 6.6 Concentration of spermatozoa recovered from patients' semen samples by the VMP method. The result shown is the mean ± standard deviation for

concentration of recovered spermatozoa from 20 semen samples of the patients with oligoasthenoteratozoospermia.

In the case of oligoasthenoteratozoospermic sample a weak correlation between the concentration of sperm in semen and the number of spermatozoa after VMP was observed (r=0.45). That means that when a sample with low concentration of sperm in semen is processed by the VMP method it is difficult to predict the penetration rate using information about the number of cells only. However, even with the poor samples, it is still possible to get motile spermatozoa in the fraction after the VMP method. On average, 7% of spermatozoa from an oligoasthenoteratozoospermic sample were able to pass through viscous medium and get into the drop of STF.

6.4.2 Influence of motility of spermatozoa in semen and penetration rate

The average percentages of total and progressive motile sperm were significantly increased after the processing semen by the VMP method from 25.3% to 85% and from 13.5% to 45% respectively. Motility plays an important role in penetration ability of spermatozoa in the VMP system (Chapter 4). The relationships between percent of total motile sperm and percent of sperm that were able to pass through viscous medium for donors' and patients' are presented in the Figures 6.7 and 6.8 respectively. The correlation between the percentage of total motile sperm and penetration rate in the oligoasthenoteratozoospermic samples (r=0.89) was stronger than the same correlation for normozoospermic samples (r=0.8).

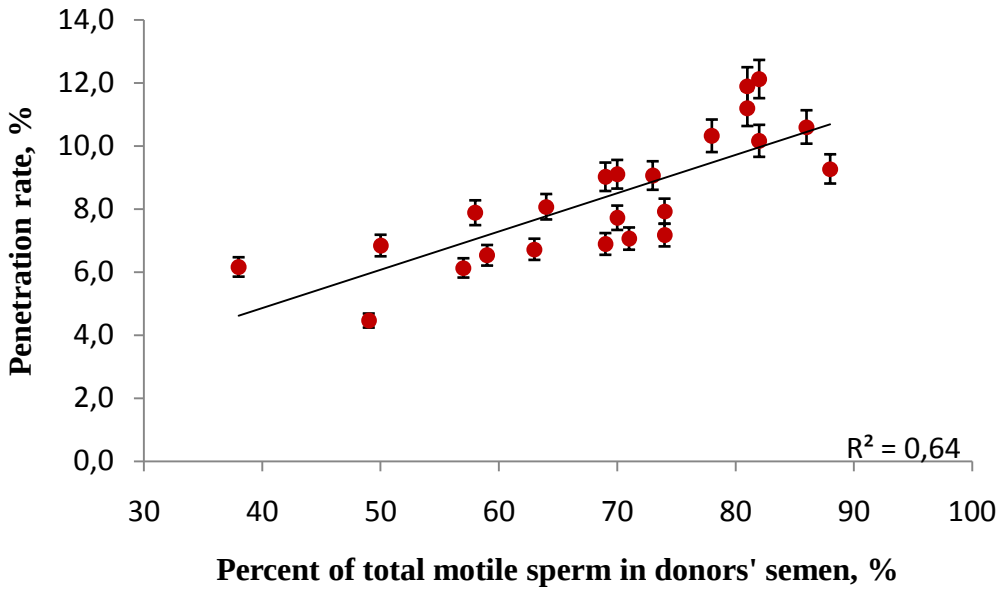


Figure 6.7 Relationship between percentage of total motile spermatozoa in donors' samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 23 semen samples.

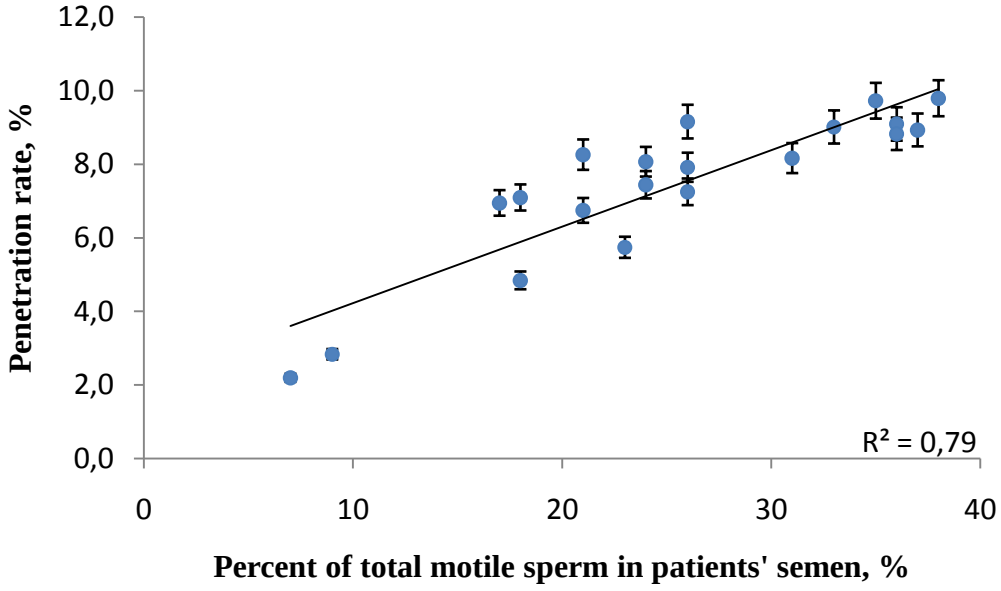


Figure 6.8 Relationship between percentage of total motile spermatozoa in patients' semen samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 20 samples obtained from the patients with oligoasthenoteratozoospermia.

The relationships between percent of progressive motile sperm and percent of sperm that were able to pass through viscous medium for donors' and patients' are presented in the Figures 6.9 and 6.10 respectively.

The correlation between the percentage of progressive motile sperm and penetration rate in the oligoasthenoteratozoospermic samples (r=0.7) was also stronger than the same correlation for normozoospermic samples (r=0.5). Therefore, sperm motility is more important for penetration ability in oligoasthenoteratozoospermic samples than in normozoospermic samples.

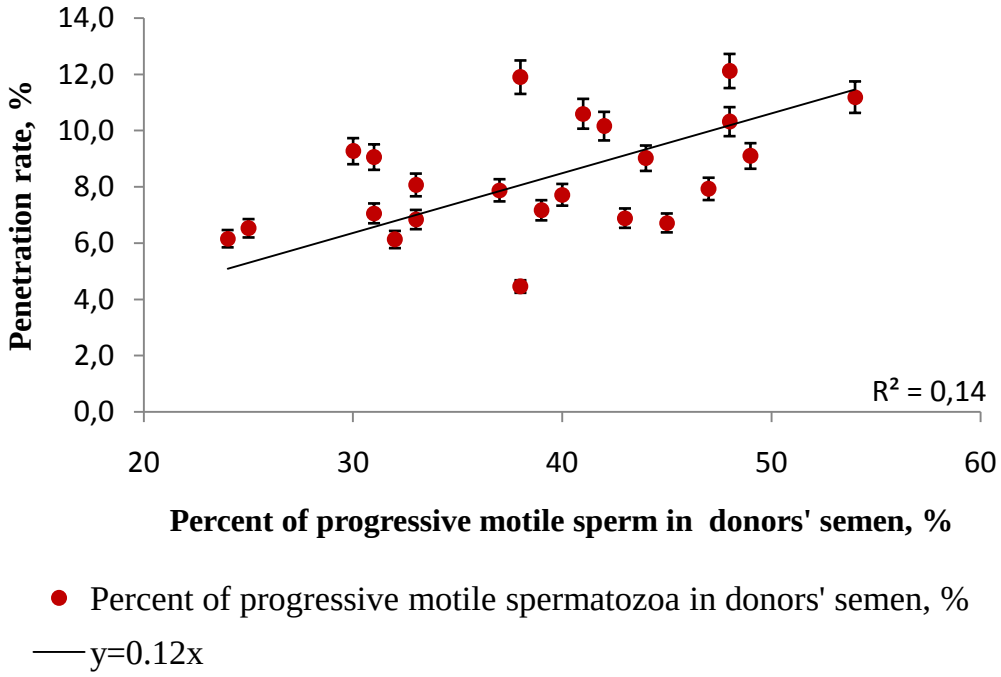


Figure 6.9 Relationship between percentage of progressive motile spermatozoa in donors' samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 23 semen samples.

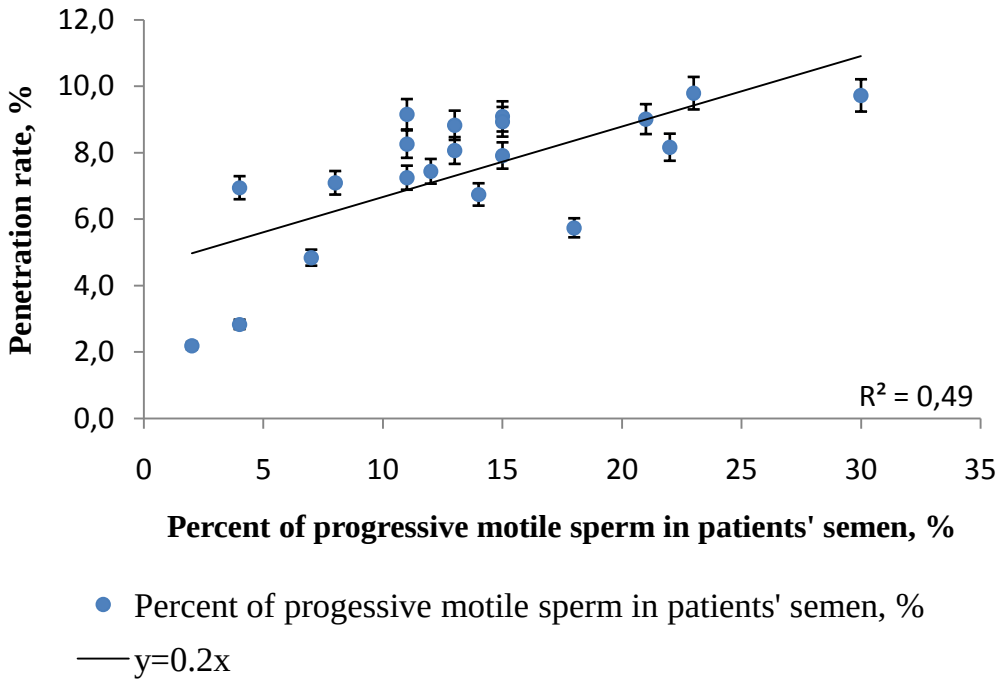


Figure 6.10 Relationship between percentage of progressive motile spermatozoa in patients' semen samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 20 samples obtained from the patients with oligoasthenoteratozoospermia.

6.4.3 Influence of morphology of spermatozoa in semen and penetration rate

The average percent of normal sperm was significantly increased after the processing by the VMP method from 2.5% to 5.5%. Although sperm with all types of abnormalities (head, midpiece, tail and cytoplasmic droplets) were able to pass through viscous medium, their frequency was significantly decreased ($P<0.05$, Fig.6.11).

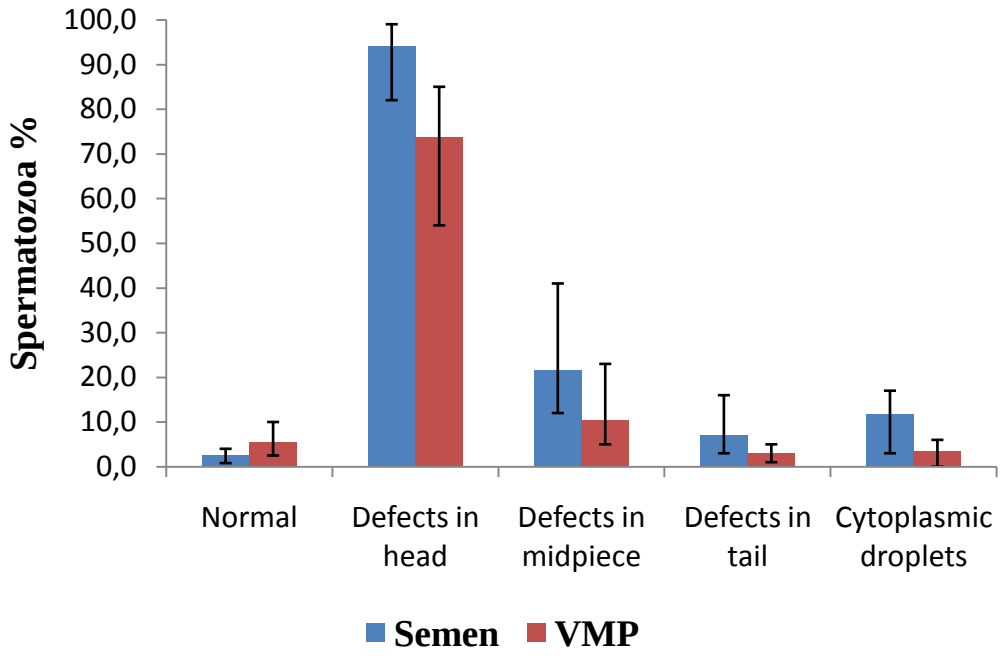
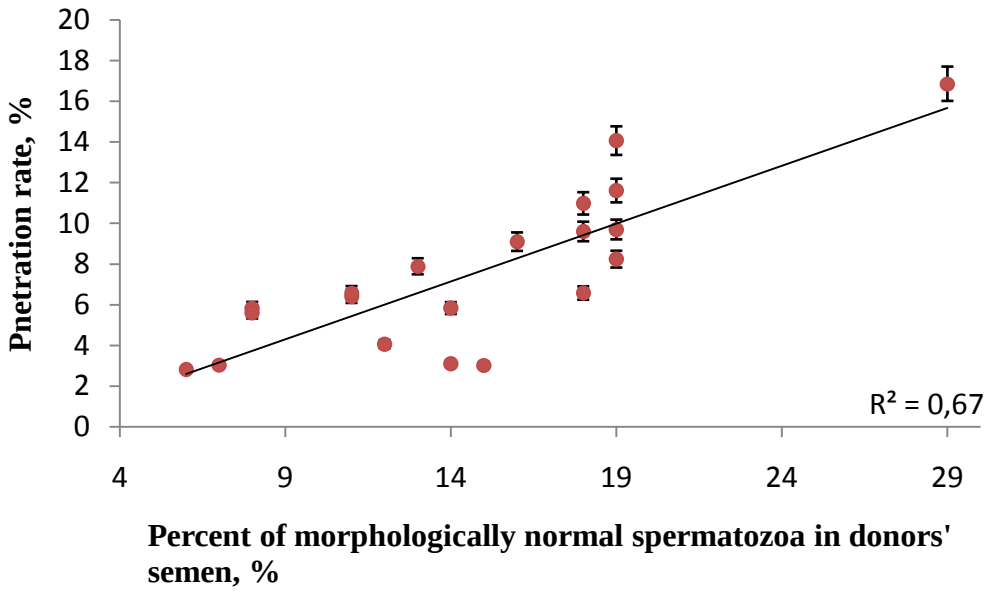


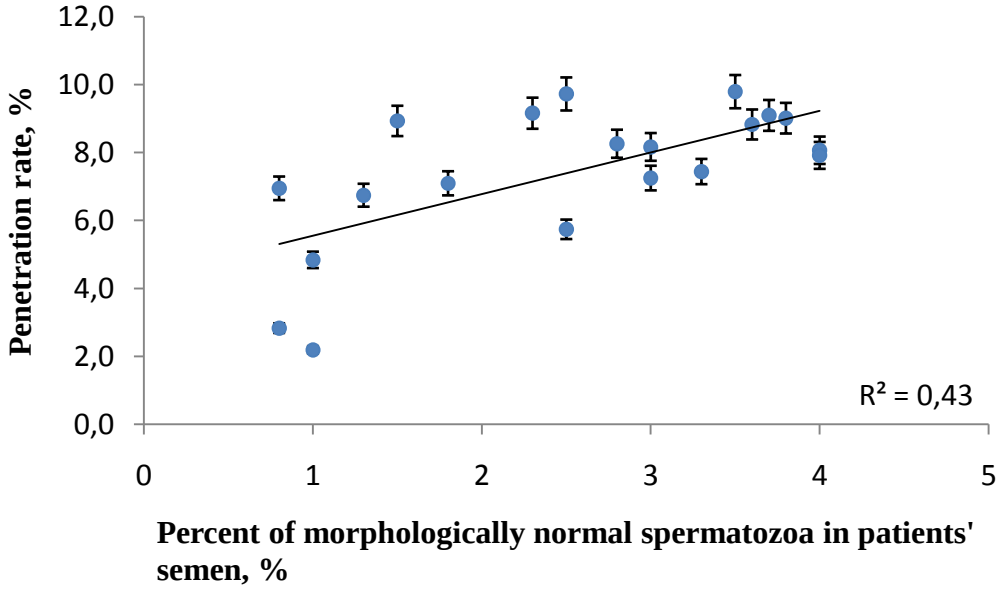
Figure 6.11 Average percentages of normal spermatozoa and the percentages of different defects of sperm in patients' semen, and sperm recovered by the VMP method (20 cases). Each vertical line indicates ± standard deviation about the mean. Statistical difference ($P<0.05$) was observed between the spermatozoa in semen and after preparation.

The relationship between percent of morphologically normal spermatozoa and percent of spermatozoa that were able to pass through viscous medium for donors is presented at Figure 6.12. High correlation was noted for the oligoasthenoteratozoospermic samples with low percent of morphologically normal spermatozoa (Fig. 6.13). Although spermatozoa with normal morphology penetrate the viscous medium more effective than those with abnormalities, some abnormal sperm were able to get through the viscous line.



● Percent of morphologically normal sperm, % ——y=0.57x

Figure 6.12 Relationship between percentage of morphologically normal spermatozoa in normozoospermic semen samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 20 donors.



● Percent of morphologically normal sperm, % ——y=1.22x

Figure 6.13 Relationship between percentage of morphologically normal spermatozoa in patients' semen samples and penetration rate in the VMP method. The result shown is the mean ± standard deviation for concentration of recovered spermatozoa from 20 patients with oligoasthenoteratozoospermia.

6.5 Discussion

The primary aim of this chapter was to test the capacity of VMP method to recover sperm from the on samples obtained from the patients with oligoasthenoteratozoospermia, i.e. the samples with a concentration of spermatozoa less 15 M/ml, total and progressive motility of sperm less than 40% and 32% respectively and morphologically normal forms less 4%. The results showed that even in these semen samples, there was a group of spermatozoa that had a capacity to pass viscous medium. On average, 7% of spermatozoa from semen were recovered by the VMP method. This value varied from 2% to 10% depending on motility and morphology of sperm in semen sample. A significant correlation ($P<0.05$) between the recovery capacity of the VMP method and sperm total ($r = 0.89$) and progressive ($r = 0.7$) motility and morphology ($r = 0.66$) was observed. Weak correlation ($r = 0.45$) was observed between the concentration of sperm in semen and the recovery ability of the VMP method. This phenomenon can be explained by the different number of motile spermatozoa in the semen samples with relatively equal concentrations.

The results demonstrate the increased frequency of normal forms of sperm that were recovered by the VMP. The similar effect was observed with the semen obtained from the donors. Moreover, the efficiency of sperm penetration was significantly correlated ($r = 0.66$) with the proportion of normal sperm in donors' and patients' semen samples. Therefore, the morphology status of spermatozoa in semen should allow prediction of their penetration capacity and recovery rate of the VMP method.

According the results presented in 3.5, extending the time of processing of the VMP method, i.e. time of incubation of the dish, does not allow a significant increase in the sperm concentration in the yield. It is possible to assume, that spermatozoa in semen can loose the ability to penetrate through seminal plasma and viscous medium after a long period of time (>3 hours). Moreover, there is a risk of high sperm DNA fragmentation after incubation for 2 h (Nabi *et al*., 2013). Thus, increasing the time of incubation should not be used, especially for oligoasthenoteratozoospermic samples, when the fragmentation of sperm DNA is usually rather high.

In the cases when concentration of spermatozoa is less than 2 M/ml instruments such as CASA and FLUOstar Omega cannot be applied because of technical difficulty in accurate measurements or low sensitivity. Probably, single-cell calcium imaging or sperm chromatin dispersion test can supply with accurate data about functional characteristics of spermatozoa that were able to pass through viscous medium.

In summary, this chapter proposes that the VMP method can be potentially applied for processing semen samples obtained from the patients with oligoasthenoteratozoospermia. Although the examination of recovered spermatozoa was limited with our instruments, the previous experiments of this research performed on donors allow the assumption that these spermatozoa can potentially produce quality embryos. This concept will be further discussed in Chapter 7.

Chapter 7 General Discussion and Potential Experiments in Future

This research is focused on the very important problem of preparing semen and sperm selection for IVF/ICSI. Currently, there are no proven methods or techniques for selecting sperm for IVF or ICSI after they were isolated from the semen. In most IVF laboratories the main method of fertilization is ICSI, as described by Palermo in 1992 (Palermo *et al.*, 1992). However, the method of sperm selection has not significantly changed since 1992. The only criteria for sperm selection in ICSI are sperm morphology and motility. These characteristics cannot indicate the DNA integrity of the spermatozoon. Sperm with fragmented DNA can fertilize eggs with the same efficiency as sperm without DNA fragmentation; but, if a sperm with DNA fragmentation is used, embryo quality will be low and critical genes can be damaged when the paternal and maternal genomes are combined at day 3 of embryo development (Henkel *et al*., 2003; Jurisicova *et al*., 1996). That might be one of the reasons why the average blastocyst rate is only 50%, although an average fertilization rate is 80% or even 100% in some cases. In IVF, the quality of the oocytes, culture conditions, and experience of the embryologists are the significant factors that influence the success of the output. For the last 20-30 years a large improvement was achieved in embryo culture, cryopreservation of embryos and gametes, and evaluation of embryo quality. However there was almost no progress in sperm selection for IVF/ICSI. Although there are several techniques for detecting sperm DNA fragmentation or aneuploidy, such as TUNEL, FISH, Comet Assay, these are invasive and do not work in real time. Currently, there are three methods of sperm selection that can be routinely used in the IVF lab: intracytoplasmic morphologically selected sperm injection (IMSI, Bartoov *et al*., 2003), motile

sperm organelle morphology examination (MSOME, Bartoov *et al*., 2001) and physiological intracytoplasmic sperm injection (PICSI, Huszar *et al*., 2007). IMSI and MSOME are based on a more detailed study of morphology. During PICSI the embryologist selects sperm that were able to bind to the hyaluronan hydrogel coated dish. These methods are technically more difficult than routine ICSI, they are expensive, they take a lot of time, and their efficiency is still debated. Moreover, it is still necessary to isolate sperm from seminal plasma to perform these methods. During this preparation it is possible to damage the sperm or lose the fraction of sperm with the best fertilization ability.

The primary aims of this thesis were:

- The development of a novel physiological method for sperm selection that will allow for the recovery of sufficient number of spermatozoa for IVF.
- Evaluation of the characteristics of spermatozoa recovered by the VMP method (concentration, motility, and morphology).
- Evaluation of the sperm characteristics that influence the penetration and recovery of sperm in the VMP method.
- Comparison of the characteristics of spermatozoa recovered by VMP and swim up.
- Assessing the functional characteristics of spermatozoa recovered by the VMP method.
- Examining if the VMP method can be applied to patients with severe semen abnormalities.

A lot of time was dedicated to designing the VMP method, to test different media, to determine configurations of the dish and processing conditions that produce repeatable and reliable results. Another problem was to choose the characteristics of control and study groups of spermatozoa that can be compared with available instruments. After the design of the new method was established, it was shown that it is possible to recover a clean fraction of highly motile spermatozoa for IVF/ICSI by the VMP method (Chapter 4). The concentration of sperm in the recovered fraction was statistically lower than the concentration of sperm recovered by swim up. For donors, on average, 9% of spermatozoa from semen were able pass through viscous line. In swim up, on average, 32% of spermatozoa migrated into the overlaid medium. This result indicates that although all motile sperm in the drop of semen have the potential to penetrate through the viscous medium, not all of them are capable to do that. It was discovered that spermatozoa recovered by the VMP method have different motility characteristics in comparison with spermatozoa recovered by swim up. Spermatozoa recovered by the VMP method have statistically higher amplitude of lateral head displacement (ALH). The results of the experiments also showed that ALH is one of the most significant parameter that determines the efficiency of viscous medium penetration. The correlation coefficient between ALH of spermatozoa in semen and their penetration ability was 0.76. It is just 0.04 lower than the correlation rate of the most significant parameter – total motility. Spermatozoa with high ALH recovered by the VMP method may potentially increase the fertilization rates in IVF, because of their higher ability to penetrate the cumulus cells and to bind with the zona pellucida of the oocyte.

With respect to Chapter 5, morphology and functional characteristics of spermatozoa recovered by the VMP method and swim up are not the same. Spermatozoa after penetration through the viscous medium have better overall morphology than spermatozoa recovered by swim up. The teratozoospermia index (TZI) of spermatozoa recovered by the VMP method was significantly better ($P<0.05$) than the TZI of spermatozoa recovered by swim up. After preparation by the VMP method, a significant reduction ($P<0.05$) of spermatozoa with defects in the midpiece was observed. It is possible to assume that viscous medium is a very efficient barrier for spermatozoa with defects in the midpiece, which is the site of mitochondria in sperm and responsible for sperm motility. It is interesting that spermatozoa recovered by the VMP method had a greater capacity to bind to hyaluronic acid than sperm after swim up. With respect to previous research (Mokánszki *et al*., 2012), this suggests that spermatozoa recovered by the VMP have less chromosomal abnormalities and a more mature structure, which is potentially important in ICSI when only one spermatozoon must be chosen and injected into the oocyte. Results presented in the Chapter 5, indicate that the probability of selection of DNA-damaged spermatozoa for ICSI after the VMP method will be reduced. Sperm DNA fragmentation might not only be a cause of poor embryo development and low implantation rate (Robinson *et al*., 2012), but also an increased risk of childhood cancer in the offspring (Aitken *et al*., 1998).

The samples obtained from the patients with oligoasthenoteratozoospermia were also tested by the VMP method (Chapter 6). Even if a very poor sample is processed by the VMP method, it is still possible to recover enough spermatozoa for ICSI. As soon as motile sperm are present in the ejaculate, some of them will

be able to penetrate through the viscous medium and can be recovered. The intracellular Ca^{2+} response level was assessed by fluorometric measurements. This sperm characteristic significantly correlates with fertilization rate in IVF and has a good prognostic value, but also has the limitations of a minimum number of spermatozoa required to be loaded into the device and thus could not be used with the patients' samples. A better comparison between the functional characteristics of sperm from subfertile patients would be gained through detailed analysis using single cell imaging of spermatozoa.

In Chapter 1, a table with advantages and disadvantages of current methods of sperm preparation was shown. Now, after testing the VMP method, it is possible to summarize the advantages and disadvantages of this method.

The advantages:

+ Simple and gentle separation method
+ Clean fraction of motile sperm with improved morphology is recovered
+ Higher percentage of HA-bound spermatozoa
+ Relatively cheap
+ Easy to perform

The disadvantage:

– The fertilization ability of recovered spermatozoa has yet to assessed
– Not the whole ejaculate is processed
– The yield is lower than in swim up

Besides the main possible application of the offered technique – to recover sperm for IVF/ICSI, the VMP method can also be used for research of sperm motility,

chemo- and thermotaxis, sperm competition or to observe the effect of different drugs on sperm motility. The VMP method as a model of the female cervix can be a powerful instrument for simulations of sperm behavior *in vivo*.

Another interesting question that was not covered in this research: what makes the sperm move through the line of viscous medium? Movement of spermatozoa in this system seems random: there are no chemoattractants or physical influence (reotaxis) on sperm. Also, no strong correlation was observed between the percentage of progressive motile sperm in semen and penetration rate in the samples obtained from the donors.

Currently, it is not possible to apply the VMP method in IVF lab and fertilize the human oocytes with recovered sperm. This experiment requires the special ethical approval by the Human Fertilization and Embryology Authority (HFEA) which takes at least 6 months to obtain. Animal gametes are not an appropriate surrogate. First, it was found that ICSI, as applied for fertilization of human oocytes, has very low success in animal models, e.g. mice. Although hamster oocytes can be used to study fertilization and pronuclear development after ICSI, their utility in studies is limited because of the difficulty in culturing the hamster embryos. Secondly, media which is used in the VMP method for recovering human spermatozoa cannot be the same for animal sperm. Thus, even if it would be possible to adjust the VMP method for animal model and design the experiment, the obtained results cannot be effectively replicated for humans. Therefore it is important to apply this novel technique on human gametes and carry out the full IVF cycle together with traditional sperm preparation as a control. In this experiment the oocytes from one donor will be split and fertilized

with sperm recovered by the VMP method and conventional sperm preparation. This approach will directly show if there is an improvement in fertilization rate, embryo development and/or implantation rate when the VMP method is used for selecting sperm. This experiment will be carried out in the near future.

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Appendix

8.1 Questionnaire for sperm donors

PRINT NAME……

Signed……. Date ……. /…../…….

SAMPLE DETAILS

Donor number: D……...

Date ……. /…../…….

Time of production……:…….

Total sample collected: YES / NO

Time of last/previous ejaculation (please circle applicable):

Mon	Tue	Wed	Thu	Fri	Sat	Sun
am/pm	am/pm	am/pm	am/pm	am/pm	am/pm	am/pm

Comments: (e.g. recent illness/medication/need to see a member of research team)

8.2 Consent form for patient/donor participation in research

ASSISTED CONCEPTION UNIT

NHS TAYSIDE

WARD 35

NINEWELLS HOSPITAL

DUNDEE DD1 9SY

Direct line (01382) 632111

Fax (01382) 633853

CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

Title of research: Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVFsuccess.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed…….

Name (block capitals)……..

Date……

Witnessed……(name)……Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, e.barratt@dundee.ac.uk

8.3 FastRead 102

FastRead 102 is a 10-chamber counting grid with integral cover slip (fig. 8.1). Each counting chamber consists of ten 4x4 grids. The volume above each 4x4 grid is 0.1 µl. The concentration (counts/ml) is given by:

$$\frac{Total\ counts}{Number\ of\ 4x4\ grids\ counted} \times 10^4$$

Figure 8.1.FastRead 102 counting chambers (photo by manufactory).

Slide characteristics:

Overall slide dimensions	85 x 40 mm
4 x 4 grid dimension	1 x 1 mm
Sample chamber depth	0.1 mm
Sample chamber volume	7 µl

8.4 Papanicolaou staining protocol for fixed smear

Solution	Duration
Ethanol 80%	30 seconds
Ethanol 50%	30 seconds
Purified water	30 seconds
Harris's haematoxylin	4 minutes
Purified water	30 seconds
Acidic ethanol (5% acetic acid + 95% ethanol)	4–8 dips
Running cold tap water	5 minutes
Ethanol 50%	30 seconds
Ethanol 80%	30 seconds
Ethanol 95%	15 minutes
G-6 orange stain	1 minute
Ethanol 95%	90 seconds
EA-50 green stain	1 minute
Ethanol 95%	1 minute
Ethanol 100%	30 seconds

8.5 Influence of the media on the motility characteristics of spermatozoa

Components	Concentration (mM)				
	Mortimer	Aitken	Visconti	Irvine Scientific	NCB
NaCl	106	95	97.8	97.8	97.8
KCl	4.69	4.6	4.69	4.69	4.69
$Na_2HPO_4 \cdot 2H_2O$	1.48	-	-	-	-
$NaHCO_3$	10	25	4	25	-
$MgSO_4 \cdot 7H_2O$	0.2	1.2	0.2	0.2	0.2
Glucose	5.55	5.6	2.78	2.7	2.78
$CaCl_2$	-	1.7	2.04	2.04	2.04
Na pyruvate	1	0.27	0.33	0.33	0.33
Na lactate	-	44	21.4	21.4	21.4
BSA	3%	0.3%	0.3%	0.3%	0.3%
HEPES	-	-	-	-	25

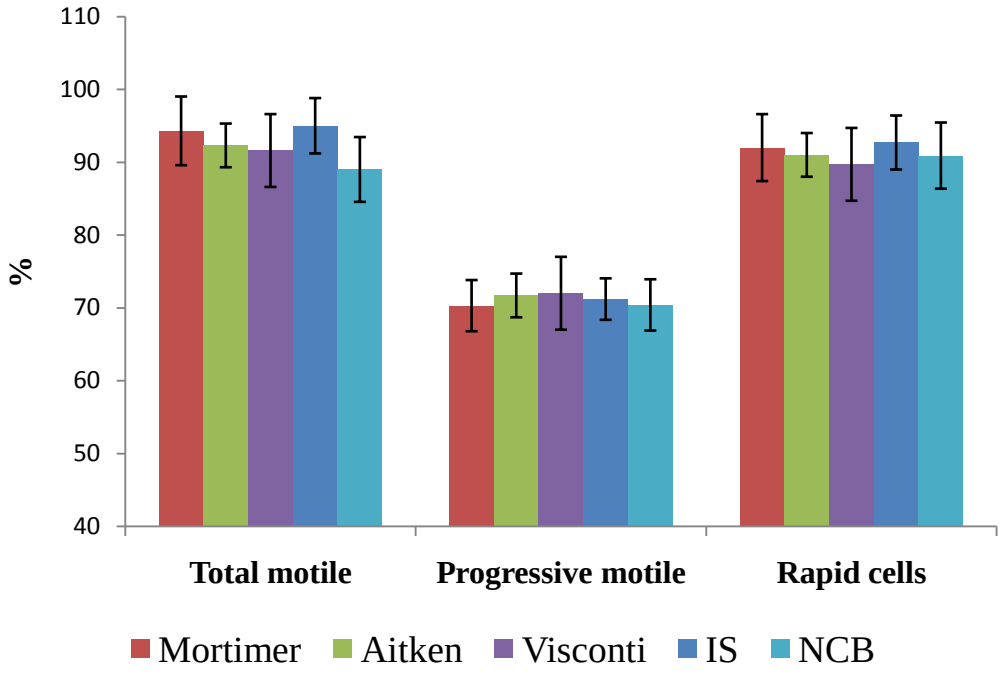

Figure 8.2 Average percentages of total motile, progressive motile and rapid cells after 60 minutes of incubation in three different media. Each vertical line indicates ± standard deviation of individual readings about the mean. No statistically significant difference was determined (n=4; P=0.68, P=0.62, P=0.63 respectively).

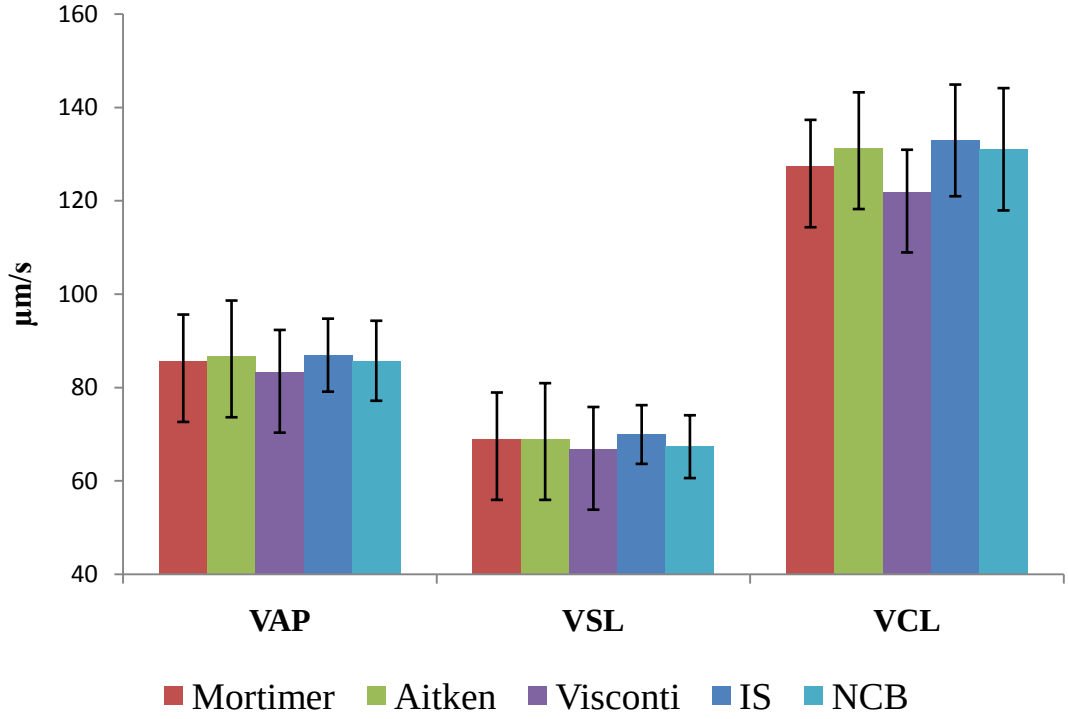

Figure 8.3 Average VAP, VSL and VCL of spermatozoa after 60 minutes of

incubation in three different media. Each vertical line indicates ± standard deviation of individual readings about the mean. No statistically significant difference was determined (n=4; P=0.61, P=0.64, P=0.53 respectively).

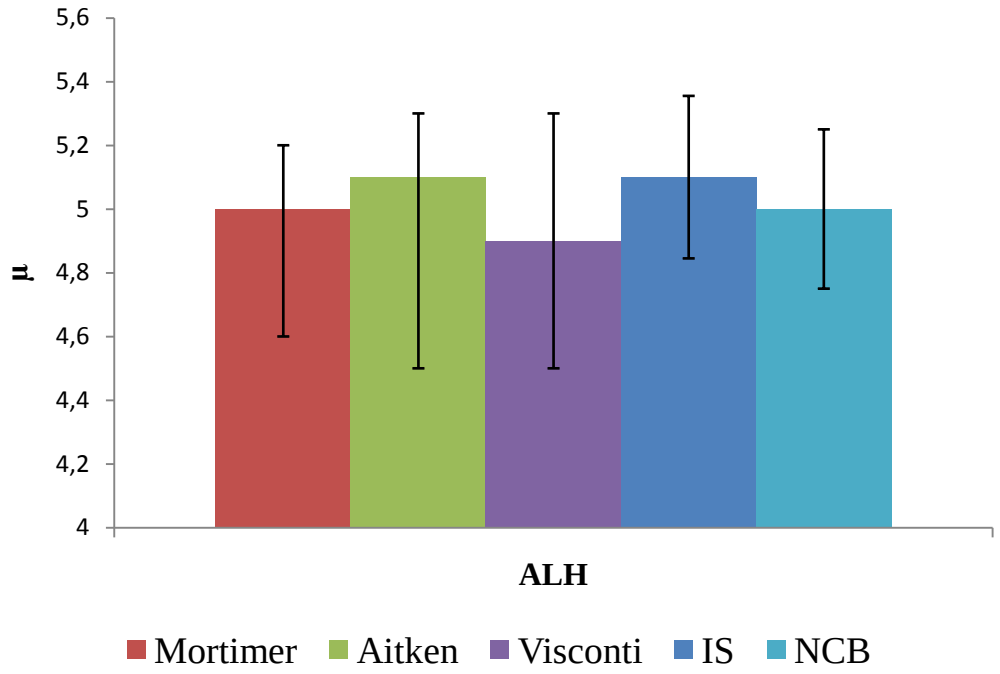

Figure 8.4 Average ALH of spermatozoa after 60 minutes of incubation in three different media.Each vertical line indicates ± standard deviation of individual readings about the mean. No statistically significant difference was determined (n=4; P=0.51).